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The next generation of weapons

IN THE late 1960s there was widespread debate in the United States on the advisability of installing defences against intercontinental ballistic missiles (ICBMs). At that time the intentions were to install radars around sites to be defended and to fire nuclear-armed rockets at any incoming missile. A defending rocket would not necessarily have to score a direct hit on an attacker—a nuclear explosion in its vicinity would suffice.

Such an anti-ballistic missile, or ABM system would have cost an immense amount and could have hardly guaranteed 100% protection. Further, its very existence would have been an encouragement to the nuclear superpowers to find means of rendering it ineffective. On the other hand, even a partial defence of a major constituent of a nation's deterrent was not to be lightly dismissed. In the event, after long and highly-informed public technical discussions, it became clear that neither superpower was strongly committed to ABM, and a bilateral treaty was signed in 1972 severely restricting deployment.

Military technology has not stood still, however, and new ways of attacking targets such as ICBMs have recently been the subject of a growing amount of interest. Are lasers or particle beam weapons a serious option? Research is being carried out in the United States and almost certainly in the Soviet Union to try and answer this question, and recently debate has moved into the public arena, notably with Richard Garwin's article on beam weapons in the October 1978 issue of the *Bulletin of the Atomic Scientists*, and with a series of articles in *Aviation Week*, also in October, on beam weapons research.

A report has recently been issued by MIT's Program in Science and Technology for International Security (*Particle Beam Weapons*, obtainable from the Department of Physics, MIT). In it, a study group reports in technical terms on the pros and cons of beam weapons in the environments both of space and on the ground.

A beam weapon damages its target by cracking its outer shell, by damaging the electronics or by exploding the warhead's trigger. Typically, to do any or all of these things with a beam of area one square metre, of the order of 100 megajoules would have to be delivered. This is not impossible. On the ground jet engines could be used; in space 60 kilograms of high explosive detonated per second would provide the necessary energy per pulse.

Propagation is more problematical. In space, charged particles are of no use. They leave an opposite charge on the generator which attracts them back towards the gun; it is estimated that a 1 GeV, 1,000 amp electron beam with a 1-cm radius would get only a metre beyond the accelerator's exit port. Even if a charged particle beam could propagate, it would be degraded over thousands of kilometres by Coulomb repulsion, and deflected by the geomagnetic field which is imperfectly known in space.

Neutral beams don't suffer the same problems, but even so, gamma-rays and neutrons are not easily concentrated to square-metre cross-sections at thousands of kilometres. Hydrogen atoms look more promising. They can be

accelerated in a linear accelerator to several hundred MeV as hydrogen with a second electron in the 2s state, or H^- . The beam can then be aimed with a magnetic lens and the electrons stripped by passage through gas. The divergence introduced by stripping leads to a less than ideally concentrated beam, but even so the components of a hydrogen-beam system appear, in the words of the report 'physically feasible'.

Within the atmosphere, charged beams ionise air and this ionisation makes propagation possible. On the other hand the beam is degraded both in energy and cross-section by scattering. What can happen, however, is that a beam of, say, electrons depositing energy along a path through the atmosphere heats that channel, and within a small fraction of a second air density has dropped to a tenth, or even less of normal. In effect, a hole can be bored through the atmosphere, and for perhaps one-tenth of a second can permit pulses of electrons to travel relatively unimpeded. Many questions remain to be answered, but the possibility of an electron beam of a few thousand amps, consisting of ten thousand or more 100-nsec pulses, being able to damage an incoming missile 'cannot be excluded'. Nor, the group find, is there any compelling reason to conclude that exoatmospheric accelerators for H^- atoms or ground-based electron accelerators could not be built at some time in the future.

Even if the system can be made to work, however, it has to do so in conditions of war. In space a hydrogen beam might be used either against satellites or to attack ICBMs. At ground level an electron beam might be used as a terminal defence against ICBMs or, at sea, to protect a ship against cruise missiles. In all instances a direct hit is called for (which means accuracies from radars of up to a few parts in a million), and even after such a hit there may be no immediate indication of success. And if the beam misses, it seems near to impossible to determine by how much. Further, the system is open to all the jamming measures that were once discussed in the ABM debate and if the accelerator were in orbit and receiving instructions from the ground that link would be particularly vulnerable. What is more a hydrogen beam would be completely disrupted by the slightest amount of air in space, so a defensive measure could be to fire an explosion in the upper atmosphere, lifting air into space.

The ABM debate is being replayed. An immensely difficult and expensive technical mission, with serious questions at every turn, will be ventured into for fear that the other side just possibly might secure an advantage. There are powerful forces to move the programme forward and spend a lot of money; and for all we know there could be success at the end of the road. There is an arms-control means of stopping development, however. The 1972 treaty noted that if ABM systems 'based on other physical principles' emerged, discussions would be held on limiting such systems. Beam weapons clearly fall into this category. Talks should start soon, even if at present there are no plans for deployment. □



Low energy, but lots of loot in UK forecast

Energy needs in the UK in the year 2000 will be at most 3% more than in 1976, and may even be 6% less, according to a report published by the International Institute for the Environment and Development last week. The report emphasises the savings that can be made by introducing proven conservation technologies, and allows for a trebling of Britain's gross domestic product (GDP). In contrast, the present official forecast of the Department of Energy (DOEn) projects an energy growth of some 35% to 70% by 2000 after allowing for a 20% saving through conservation measures.

The IIED forecast may nevertheless not be extreme. The DOEn admitted last week that their 20% conservation figure was a rough one, and said that they had been waiting for nearly a year for a response to it. Their figure was based on consumer research by the Electricity Council and the Gas Boards, advice from the Department of the Environment on the progress of insulation of buildings, "and many other sources", but was open to revision. The IIED report would have its impact on the forthcoming Green Paper on energy futures, said a spokesman, although there was too little time to take it fully into account.

Mr Gerald Leach, the author of the

A low energy strategy for the UK (Science Reviews Ltd and IIED) £7.50. Obtainable from IIED, 10 Percy Street, London W1.



report, said last week that far from having an energy gap the UK had "a great deal of room to manoeuvre on supply options, without extreme measures". Much of the necessary conservation technology — such as heat pumps for domestic space heating and efficient internal combustion engines — will become available in the 1980s, he argued, when the devices will begin to replace existing equipment. Cost savings in running the technology will lead to its adoption by the consumer with little or no encouragement from the government, he argues. "We have found that it is astonishingly easy to save energy" said Mr Leach. "We began this work as energy pessimists, but now we are optimists".

Many of the measures are emerging as a result of the 1973 oil crisis, says the report, when it became fashionable as well as expedient to consider ways of saving energy. The effect of this work is mostly yet to come. The Royal Institute of British Architects, for example, has enthusiastically adopted conservation, an attitude which has

already resulted in buildings using half as much energy as before for only a 2 to 3% cost penalty, but building stock is replaced only slowly. In the home, lights, cookers, and heaters will soon enjoy "very substantial increases in efficiency". Small-scale heat pumps, such as the one being designed by the Gas Boards, are "at an advanced stage" and will make a dramatic impact. The motor industry is designing cars with a 50% reduction in fuel consumption. Industry uses 40% of primary energy, "but a large proportion of that is in heating poorly insulated buildings, where strict regulation is on the way" says Gerald Foley, one of the team that produced the report. Indeed the government has already adopted measures which will improve industrial conservation. "The result is that all across the board there is slack" said Mr Leach. "For example, we predict a need for only 26-30 GW by 2000, whereas the DOEn assumes 83 GW. Quite clearly there is no need for nuclear; we have assumed 4½ to 6½ GW to keep the industry in business. The breeder reactor could be postponed indefinitely." Even renewable sources were not strictly necessary: the report assumes 4-6 million tonnes of coal equivalent produced in 2000 from alternative sources, compared with 10 in the DOEn projection.

The report may, however, have assumed unrealistic substitution rates. The most uncertain element may be the cost of the new technology, and of

Britain's energy optimist

Gerald Leach, author of *A low energy strategy for the UK* (see above), may see himself as an energy optimist—but the *Financial Times* last week saw it differently. Thinking no doubt of the markets lost to the energy supply industry, the sub-editor had headlined the story about Leach's report "Bleak forecast for UK energy". But Gerald Leach's interests do not lie with big business.

Gerald Leach is 44, and left science writing and broadcasting (for the BBC and the *Observer*) in 1972. At the *Observer* he had become interested in the works of Commoner and Ehrlich, and had accumulated "shelves of fascinating books with no time to read them. As a journalist I felt I was skating over an ocean." Then he met a representative of the Ford Foundation at the Stockholm environment conference, cheque book



in hand, and his new career began.

He produced a series of reports for various bodies on energy matters, including *Energy and Food Production* (for IIED), *Fuel conservation: options for aviation* (for OECD), *Nuclear energy balances in a world with ceilings* (for IIED), and gave evidence on energy futures to the Windscale inquiry on reprocessing. He defended "Limits to growth" at the Royal Society, did "a big splash" in the *Observer*; but "I began to feel uncomfortable. Early on he realised the importance of "saturation" effects—such as a ceiling on the

number of cars, or the temperature of a house, or conservation. Growth might limit itself.

"Early in 1976 I'd finished a project and I went down to Her Majesty's Stationery Office to read all the government reports on energy. There turned out to be only 4 pages on futures." But the US was much further ahead. "My first task was to convince the Ford Foundation that we were so far behind". Then he got the grant, and the study was under way under the wing of the International Institute for the Environment and Development. "The original proposal to Ford was on alternative sources with a bit of conservation" said Leach. "Then we began to feel strongly about disaggregation", calculating energy futures by adding up all possible uses rather than using macroeconomic trends. It also emerged that previous reports were unduly moderate on conservation. The result was the low energy prediction just published. □

fuels which will affect substitution. "We will be attacked by economists" admits Leach. The report has adopted apparatus replacement rates of some 5% per year after the new technology becomes available. Thus heat pumps have a 15-20% penetration of the market by 2000. But in housing the report allows until 2010 before all houses have 4 inches of loft insulation, and cavity walls are filled.

So far, the Department of Energy response has been friendly, and mildly defensive. "My feeling is that there is a lot of work to do yet in conservation in our methodology" said a spokesman. "We are only scratching the surface . . . But has Gerald Leach only taken the most favourable answers from all his inquiries? In our last green paper we took advice from the department of transport, the oil companies, and car manufacturers among others. Most thought that 20% conservation was ambitious. Mr Leach is applying current best practice across the board, and concludes that one can increase that 2 or 3 times. That may be wishful thinking."

The team responsible for the IIED report consisted of Gerald Leach, Christopher Lewis, Frederick Romig, Ariane van Buren, and Gerald Foley.

Robert Walgate

... and the view from across the Atlantic

Forecasts of US primary energy demand in 2000 AD

(in quads = 10^{15} BTU)
(1975 consumption 75 q)

Year of forecast	Source of forecast			
	beyond the pale	heresy	conventional wisdom	superstition
1972	125 <i>Lovins</i>	140 <i>Sierra Club</i>	160 <i>AEC</i>	190 <i>Fed Power Comm'n</i>
1974	100 <i>FF (Zero growth)</i>	124 <i>FF (Tech. fix)</i>	140 <i>ERDA</i>	160 <i>Edison Electric Inst.</i>
1976	75 <i>Lovins</i>	89-95 <i>v H and W—Lovins</i>	124 <i>ERDA</i>	140 <i>Edison Electric Inst.</i>
1978	33 <i>Steinhart (for 2050)</i>	63-77 <i>CONAES panel (for 2010)</i>	95-100 <i>CONAES, DOE-IEA</i>	123-124 <i>RDE—Ralph Lapp</i>

Energy forecasts have been falling in many countries, not only the UK. Amory Lovins has communicated this table of US forecasts for the year 2000, produced by various bodies over the last six years. "Read diagonally, the matrix has considerable predictive power" says Lovins. The September 1978 Department of Energy forecasts were not known when the table was first prepared. However, there is considerable subjective judgement involved in the choice of columns and of forecasts to fit them. Read literally, the matrix predicts that the conventional 1980 US forecast for the year 2000 will be some 70 quads, less than 1975

consumption.

Abbreviations used: AEC, Atomic Energy Commission; FF, Ford Foundation; ERDA, Energy Research and Development Administration; vH and W, von Hippel and Williams; CONAES, the Committee on Nuclear and Alternative Energy Systems of the US National Research Council; and DOE, Department of Energy. In 1976 Lovins' two forecasts were private (75q) and public (95q, published in *Foreign Affairs*). In 1978 the CONAES panel used three scenarios, as did the DOE (varying with projected oil price). □

Black year for Soviet refusniks

1978 was, in general, a black year for Soviet refusnik scientists, marked by increasing harassment and the imprisonment or exile of a number of the best-known of them. However, the year ended on a note of partial triumph—in the last week of December the "Sunday seminar" for refusniks achieved its long-standing aim of an international meeting with the participation of scientists from USA, France and the UK.

Inevitably a number of the intending foreign visitors failed to receive the necessary visas. Five of the eight would-be participants from the US had their visas revoked—evoking a statement of censure from the Committee of Concerned Scientists saying that it "found it unconscionable for the USSR to seek to prevent American scientists from engaging in scientific discussions with Soviet colleagues". The conference, it claimed, was "an excellent example of the kind of unofficial, non-governmental exchanges provided for by the Helsinki Final Act."

"Non-governmental" exchanges do not fit easily into the Soviet concept of science, and previous attempts of the seminar group to hold an international meeting were less successful. In 1974, no foreign visas were issued and the leading Soviet participants were arrested. Nevertheless, the proceedings

of the "Conference that never was" were published in the regular manner, and limited foreign participation was permitted in the April 1977 meeting to commemorate the fifth anniversary of the founding of the seminar.

The apparent acquiescence of the Soviet authorities with regard to last month's meeting seem due rather to international pressure than to any change of heart. Continuing routine surveillance of the venue—the flat of Viktor Brailovskii—during the seminar, and a thorough police search with confiscation of his recent scientific material and his Lenin library ticket made this clear. A number of other intending participants suffered like harassments.

Nevertheless, the meeting itself took place. The main purpose of last month's gathering was to bring the refusniks abreast of recent Western developments.

The chosen title "collective phenomena" allowed a wide range of subjects. The US participants presented work in statistical physics and vortex motion in superfluid ^4He , while the French contributions ranged over crystal physics, axiomatic quantum field theory, one-dimensional conductors, and what one participant described as a "very pretty" result in percolation

theory. Since this last contribution required no specialist background, it was of particular value to the refusniks, many of whom have inevitably fallen irrevocably behind in their original field, and who therefore wish to extend their acquaintance with other subjects.

Local contributions were, understandably, somewhat hampered by the extremely restrictive nature of refusnik life—lack of access to experimental facilities and to new results in particular. Nevertheless, a wide range of subjects was attempted, including solitons, plasma physics, and quantum field theory. Yurii Orlov, one of the few non-Jewish members of the original seminar group, could not be present in person—he is at present serving a 12-year sentence for "anti-Soviet agitation and propaganda". A paper of his on "Wave logic", an alternative to conventional two-valued logic, more appropriate to quantum mechanics was presented in absentia.

Another gentile who has always taken a keen interest in the Seminar, is academician Andrei Sakharov. To the delight of all participants, Sakharov attended the first session. Other notable Soviet participants included Aleksandr Lerner, Grigorii Rozenshtein, Yurii Fishman, Yurii Gol'fand and Yakov Al'pert.

Vera Rich

Psychosurgery supporters sued for malpractice

David Dickson reports from Boston on a little-publicised court case that has revived debate on the psychiatric use of surgery

Two prominent supporters of the use of surgery to control the behaviour of individuals are being sued for malpractice by a former patient who, it is claimed, is now confined to a mental hospital as a direct result of the treatment that he received.

The charge of negligent treatment has been made against Dr Vernon H. Mark, Director of the Neurosurgical Service at Boston City Hospital, and Associate Professor of Surgery at Harvard Medical School, and Dr Frank R. Ervin, now Professor of Psychiatry at the University of California in Los Angeles.

Dr Mark and Dr Ervin became the centre of a fierce public controversy in 1967 when, in a letter to the *Journal of the American Medical Association*, written with Dr William H. Sweet, Professor of Surgery at Harvard Medical School, they suggested that brain dysfunction in individuals might have contributed to the urban riots that swept several American cities in the late 1960s—and that people with 'low violence thresholds' should therefore be diagnosed and treated before they contributed to further such tragedies.

Three years later, Drs Mark and Ervin published a book *Violence and the Brain* in which they wrote that they intended to stimulate a "new biologically-oriented approach to the problem of human violence" and that this should lead to a "more rational approach" to the problems of violence.

The suit claiming malpractice has been filed by the mother of Mr Leonard A. Kille, previously a research and development engineer with the Polaroid Corporation. It is being handled by the Washington law firm on which Health Secretary, Mr Joseph Califano, was once a senior partner.

While working for Polaroid, Mr Kille headed a team that was responsible for a number of patents on the land camera. He received treatment, including brain surgery, from the two defendants in 1966 and 1967 and he is generally taken to be the patient 'Thomas R.' described in *Violence and the Brain* as a "brilliant 34-year-old engineer with several patents to his credit", whose treatment Dr Mark and Dr Ervin say represented the first time they were able to demonstrate that systems in the limbic brain both start and stop attack behaviour.

According to evidence presented to a jury which is currently considering the charges in a Boston court-room, Mr Kille was referred to the Boston

General Hospital in 1966 after a history of epileptic seizures and outbursts of violent behaviour mainly directed towards his wife. He was diagnosed as having psychomotor seizures and 'personality pattern disturbance', and subsequently as suffering from temporal lobe epilepsy.

Following apparently unsuccessful attempts by the hospital to treat him with a variety of drugs, Dr Mark and Dr Ervin implanted a series of electrodes in his brain, the stimulation of certain of which relieved his aggressive symptoms. They subsequently performed an operation by making small bilateral lesions in his medial amygdala through heating the electrodes.

Immediately after the operation, according to evidence presented in the court, Mr Kille's behaviour seemed to have improved. At one point for example, he said that he 'felt like a new person'. However, shortly after his release from hospital his violent tendencies returned, and a month later he was admitted to a west coast hospital where he was described as "hallucinated, delusional and confused" and diagnosed as schizophrenic and paranoid.

A succession of hospitalisations and subsequent discharges followed, with continued signs of aggressiveness and belligerence. Mr Kille was eventually placed under permanent care at the Bedford Veterans Administration hospital, 25 miles north of Boston, where he is now confined. In the suit that has been filed on his behalf, it is now claimed that he was "rendered of unsound and unbalanced mind" as a direct result of negligent treatment by Dr Mark and Dr Ervin.

In particular, it is alleged that the surgeons failed to inform him fully of the risks and ramifications of the surgical procedures that they were recommending, and that they therefore failed to obtain his informed consent. A charge of battery resulting in permanent injury and incapacitation has been made and damages of \$2 million are being sought.

During the court proceedings, the plaintiffs have claimed that the surgeons considered that they were treating a behavioural, rather than a medical, condition (in their book they describe T. R.'s chief problem as being his violent rage), and that although the procedures they were adopting should have been described to Mr Kille and his wife as experimental this was not in fact done.

Suggesting that there might also have been a conflict of interest between medical and scientific priorities, the plaintiffs have pointed out that Dr Mark told a neuroscience seminar in December 1966 that inserting electrodes in the brain for therapeutic reasons served a dual purpose since it had "real value both to the patient and to our scientific enquiry".

They have also quoted a passage from a later printing of *Violence and the Brain* in which Dr Mark and Dr Ervin state that "Four years have passed since the operation, during which Thomas has not had a single episode of rage. He continued, however, to have an occasional epileptic seizure with periods of confusion and disordered thinking". This indicates that the surgeons were primarily concerned with altering their patient's behaviour rather than his medical condition—and that they felt they had successfully treated the former.

The defendants have replied to the various charges by saying that Mr Kille was diagnosed as suffering from temporal lobe epilepsy, which gave rise to his seizures, and that given the state of medical knowledge at the time, as well as the patient's failure to respond to more conventional treatment, the surgical procedures that they took were appropriate.

They also claim that during the meeting at which Mr Kille and his wife formally gave their consent to the amygdectomy operation, it was explained that there was roughly a one-third chance that the operation would successfully relieve his symptoms, a one-third chance that there would be some improvement, and a one-third chance that there would be no improvement or a deterioration.

Finally responding to the published description of the patient T. R., Dr Mark told the jury that this was a composite case history with detail taken from a number of patients, and that it should not be taken as describing exactly the case of Mr Kille.

The whole case has revived the debate on the ethics of psychosurgery which flourished at the beginning of the decade. Two years ago, following widespread public concern on the issue, the National Commission for the Protection of Human Subjects of Biomedical and Behavioural Research issued a report which, to the surprise and disappointment of some and the delight of others, gave psychosurgery its approval in some circumstances.

Though professing to an initial scepticism, the report drew on studies showing that a large number of

patients had been successfully relieved of pain or depressive symptoms as a result of such surgery to support the contention that it was a potentially beneficial therapy.

Others still claim, however, that although psychosurgery may be effective in alleviating symptoms such as pain and depression, this is frequently at the expense of sacrificing other aspects of the individual's identity, such as emotional responsiveness and creativity. It is also claimed that the psychosurgeons' approach reduces social behaviour to a purely biological phenomenon, thus denying a range of environmental factors which alternative forms of treatment, such as psychotherapy, can take into account.

"There is no scientific foundation for attempting to control a specific aspect of social behaviour by destroying a portion of the human brain", says Dr Stephan Chorover, Professor of Psychology and Brain Science, at the Massachusetts Institute of Technology, who has given evidence during the court hearings. "The main thrust of this approach to social problem solving is that it tries to localise the problem in 'defective individuals' and therefore embodies a decision not to search for problems in the larger social system".

Whichever way the court decision goes, it is unlikely to resolve the wider philosophical issues surrounding the use of psychosurgery, since the matters on which the jury has been asked to judge relate essentially to the adequacy of the medical procedures followed prior to and during the treatment.

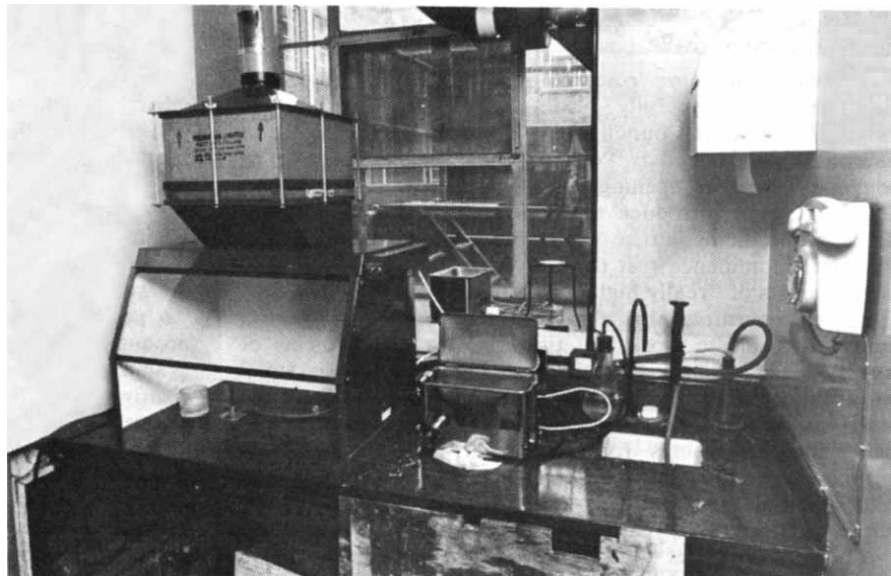
At the same time, however, it could provide a precedent for further legal actions. Already a second case is being brought on behalf of a girl who, at the age of 19 but without her consent, and after a series of electric shock treatment but minimal psychotherapy, had a cingulotomy—a lesion of the cingulum—performed by another Boston surgeon. This case is scheduled to be heard shortly. □

Scientist may sue over "Golden Fleece" award

THE Supreme Court has agreed to rule on whether Senator William Proxmire committed libel when he made a "Golden Fleece" award to a scientist engaged in a study for NASA of teeth-clenching by monkeys.

Senator Proxmire, who is chairman of the Senate Appropriations Subcommittee responsible for the budgets of both NASA and the National Science Foundation, has frequently made such awards to esoteric- or naive-sounding research projects, which he claims to be an unnecessary drain on the public purse. One recipient of the award was a research scientist, Dr Ronald R.

More dangerous smallpox labs?



Above: the smallpox laboratory at Birmingham University from which the virus escaped last August. A possible route of infection—through a cable duct—appears bottom left.

AS FAR as the World Health Organisation (WHO) is aware there are now nine laboratories in the world holding live smallpox virus; but only three of them—those in London, Hamburg and Atlanta—are known to match up to WHO smallpox laboratory safety recommendations. The laboratories at St Mary's Hospital, London and the Centre for Disease Control, Atlanta are to become WHO collaborating centres for poxvirus research; so is the Research Institute of Viral Preparations, Moscow, which is currently making modifications to come up to the WHO standard.

The Institut fur Schiffs und Tropenkrankheiten in Hamburg, the Rijks Instituut voor de Volksgezondheid, Bilthoven, the Netherlands, the US Army Medical Research Institute for Infectious Diseases and the American Type Culture Collection are all considering either destroying or transferring their viruses to one of the WHO collaborating centres. The latter three labora-

tories have not been visited by WHO. The other two laboratories currently holding smallpox virus are in Peking (which WHO has no plans to visit) and South Africa, which does not come up to standard, is storing the virus, but doing no research. Only the Centre for Disease Control in Atlanta is doing smallpox research at the moment.

The risks of laboratory infection should be seen in proportion, however. The WHO smallpox eradication unit has reduced the death rate from the disease from one million per year 10 years ago to probably zero this year. And smallpox is not an easily transmitted disease: it is not as infectious as flu or measles, for example. □

● **Correction:** six white coats per laboratory worker were recommended by the Howie report on a code of practice for the prevention of infection in clinical laboratories, not the Godber report on the handling of dangerous pathogens, as indicated in last week's leader.

Hutchinson, of Kalamazoo, Michigan, who, according to Mr Proxmire, has received more than \$500,000 in federal funding for research that includes measuring the latent aggression of laboratory monkeys under various conditions by recording electrical impulses in the jaw muscles.

Although NASA has said that the work was of particular importance in its efforts to identify aggressive personality traits in selecting crews for long space flights, Mr Proxmire called the research an outrageous example of "wasteful, extravagant, stupid spending".

A lower court rejected a libel action for \$8 million brought by the research worker partly on the grounds that he had become a "public figure" by soliciting government grants and publishing occasional articles in professional journals. Senator Proxmire also claimed legislative privilege. The Supreme Court has now agreed to hear an appeal from Dr Hutchinson, who claims that, as a result of the award, he has suffered injury to his reputation, illness, mental anguish and prospective loss of economic advantage.

David Dickson

news in brief

MRC calls for more research students despite poor job market: In a generally bleak annual report, the UK Medical Research Council (MRC) has announced it is to recruit young scientists "despite the current shortage of long term career openings in the universities". However, the MRC will introduce three new measures to alleviate some of problems of the most able research workers and to bolster confidence that the UK will provide opportunities for scientists of "really high ability to develop their talents". The new measures are: a salary grant scheme to permit university staff to work full time on research projects of a very high standard; a similar scheme to provide a small number of clinical research posts for clinical academic staff in the country's medical schools; and five-year renewable fellowships for "particularly able people" to bridge the period between a researcher's first employment and subsequent permanent post. The MRC aims to appoint three such fellows a year, building up to a total of 15.

The report makes no mention of scientists currently in employment on short term contracts. According to a recent AUT estimate there are 5,000 researchers in the UK on short-term contracts funded by the MRC and an additional 1,700 registered as unemployed with the Department of Employment. (*Medical Research Council Annual Report 1977-78* (HMSO) £3.75).

Czech Chartist jailed for nine months: Dr Jaroslav Sabata, a former professor of psychology and one of the three spokesmen of the Czechoslovak Charter 77 human rights group, was sentenced last week to nine months' imprisonment on the formal charge of "insulting a police official". Dr Sabata, who was a prominent Communist Party official during the "Prague Spring" of 1968 and who has since served five years of a seven year prison sentence for dissident activities, is a fervent advocate of closer links between the human rights movement in East European countries. His arrest last October near the Polish-Czech frontier took place during a series of meetings between the Czech Chartists and the Polish "Committee for Social Self-Defence". Authorities on both sides of the frontier tried to prevent the meetings.

During the trial, the procurator described Sabata as a "criminal" whose activities threatened the whole Czechoslovak people—a clear indication that his real offence in official eyes is his constant campaigning for personal, political and academic freedom.

Australia restructures its science planning: Following the report of the first inquiry into the functioning of the Commonwealth Science and Industry Research Organisation (CSIRO) since 1949, the Australian Government has announced a restructuring of CSIRO's top management to "facilitate closer liaison between researchers and users of research results, particularly industry". Described as an "update" rather than a "shake-up", the move organises the 37 CSIRO divisions with their staff of 7,000 into five institutes, each with a new director. The old executive structure of five full-time members and four part-timers has been replaced by a new policy-making body composed of three full-time members plus the directors of the new institutes. The identity of the 37 divisions has been preserved and the autonomy of CSIRO is not affected.

The new groupings and their directors are: Institute of Animal Sciences (Dr K. A. Ferguson); Institute of Biological Resources (Mr M. V. Tracey); Institute of Earth Resources (Mr I. E. Newman); Institute of Industrial Technology (Professor Emeritus H. W. Worner); Institute of Physical Sciences (director to be appointed); and the Bureau of Scientific Services (Mr S. Lattimore).

Ustinov on Einstein: Peter Ustinov (right), actor, writer and director, will on 14 March introduce and narrate a special two-hour BBC television programme celebrating the centenary of Einstein's birth. Ustinov, for whom mathematics and science were a total mystery when he was at school, has proved to be "a perfect student", according to producer Martin Freeth. The programme will concentrate on general relativity, the most difficult of Einstein's theories and features experimental and theoretical physicists Kenneth Brecher, Wallace Sargent, Irwin Shapiro, Roger Penrose, John Wheeler, Sidney Drell and Dennis Sciama. Written by Nigel Calder, the programme was filmed on location at the 107 inch reflecting telescope at the McDonald Observatory of the University of Texas. The programme will also use supporting film from other research centres showing current research results that support Einstein's theory.



Expanding Third World does not bring unemployment: Developing countries no longer simply sell raw materials in exchange for manufactured goods but are becoming manufacturing nations in their own right, and their increase in productive capacity is now being felt in the industrialised countries. Between 1972 and 1976, imports of manufactured goods to EEC countries increased from \$27 billion to \$76 billion. Experts consulted by the EEC have now cautioned against imposing trade barriers, saying that European industry must be adjusted to make it more competitive by "transferring production from stagnant or declining industrial sectors to those with a more promising outlook."

The effects on employment, while potentially serious, are held by EEC experts to be insignificant. Industrial employment began to decline in 1965. Using the UK as an example, the job losses attributable to Third World competition are 0.05% in textiles, 0.4% in the shoe industry, 0.8% in cotton fabrics and 1.7% in clothing. The real problem, say the experts, is the shifts caused by the new international division of labour which is causing job changes for European workers rather than unemployment *per se*. (*euroforum*, 12 December, 1978.)

Miners oppose nuclear power station near Newcastle: The National Union of Miners (NUM) is mounting a campaign to oppose a Central Electricity Generating Board plan to build a nuclear power station at Druridge Bay, near Blyth. Mr Sam Scott, secretary of the Northumberland region of the NUM, said: "The CEGB is proposing a nuclear plant on top of one of the biggest combined coal pits in Europe and parallel to parkland held by the National Trust. It's stupid".

But Mr A. D. Nash, of the CEGB's planning department, said the plan was "only an exploration of options". There was no commitment to nuclear power. CEGB projections based on an annual 2%–3% growth in demand indicated that new capacity would be needed by the end of the 1980s which could be supplied either by coal or nuclear power. With the six-year lead times necessary to build new plant, it was necessary to explore the options now, Mr Nash said. The miners, along with the National Coal Board, are seeking assurances from the CEGB that coal consumption in the area will not be cut.

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Why the world's deserts are still spreading

The fight against desertification is being held up while the politicians wrangle, writes Anil Agarwal

THE hopes generated by the United Nations Conference on Desertification (UNCOD) for achieving zero desert growth by the turn of the century, seem to have all but disappeared in just over a year. UNCOD, held in Nairobi in autumn 1977, was widely hailed as one of the most successful UN conferences of the decade. It created an unprecedented awareness about the magnitude of the growing deserts problem, prompting even US President Jimmy Carter to declare that desertification is "one of the most serious problems of our age." It also made a very significant intellectual contribution, unlike most UN conferences, by increasing the understanding about the processes of desertification.

Now, however, there is disillusionment, inaction and fading of interest, according to Professor Mohamed Kassas, Professor of Plant Ecology at the University of Cairo, who played an important role in the organisation of UNCOD. Professor Kassas, who was recently elected President of the International Union of Conservation of Nature and Natural Resources (IUCN), claims that neither the governments of the countries affected by deserts, nor the rich donor countries who were expected to provide substantial funds, have yet shown any serious commitment to combatting desertification.

Most of UNCOD's recommendations were directed towards national governments. It was hoped, for instance, that each country would set up an official focal point to coordinate its desert-related activities. In many developing countries, however, there is often such a gap in communications that one arm of the government is unaware of what the other is doing. While one ministry may be preparing programmes to control deserts, others may be pushing through agriculture and animal husbandry projects that result in increased desertification. The need for coordination is thus important. However, no African government has yet set up such a focal point.

The plans of the United Nations Environment Programme (UNEP) to

set up a consultative group for desertification control (DESCON) have also not proceeded very far. UNEP brought together possible donor and recipient countries in May 1978 for discussions. These countries agreed in principle that a mechanism like DESCON is required to mobilise resources for anti-desertification programmes in developing countries. But few of them have yet officially written to UNEP even to confirm their membership of such a group. In these circumstances, the substantial financial assistance that is required to carry out the numerous programmes suggested at UNCOD, is quite unlikely to materialise.

Politics, both national and international, lies at the heart of this lack of interest. At the international level, there is the growing disillusionment of the rich Western countries with the United Nations system, which is reflected in their growing keenness to channel financial assistance directly to recipient countries through bilateral agreements, instead of through the United Nations agencies. This reaction is setting in partly because of the inefficiency and sluggishness of the UN agencies, but even more so because of their involvement with laudable but contentious issues like the New International Economic Order, which irritate many influential politicians in the West. The same rich countries which

are fighting shy of making any commitment to anti-desertification programmes through UNEP have pledged over \$2,000 million for the development of the Sahel countries through the Club du Sahel, a non-UN organisation.

At the regional level, it is the lack of political will amongst Third World governments to overcome their political differences with each other that bedevils anti-desert programmes. Realising that neither deserts nor desert people, like nomads, recognise political boundaries, UNCOD had tried to formulate solutions to the desert problems with a transnational perspective. UNCOD therefore proposed giant transnational projects like a green belt across North Africa, another green belt across the Sahel, a joint livestock management programme in the Sudano-Sahelian countries, a joint groundwater management programme covering northeast Africa and the Arabian peninsula, and two projects to monitor desert creep in southwest Asia and south America; and it expected feud-ridden countries like Egypt and Libya, Libya and Sudan, Morocco and Algeria, Arab Yemen and People's Yemen, India and Pakistan, and Chile and Argentina, to co-operate in their implementation.

UNEP had indeed taken the bold step to prepare feasibility studies for these transnational projects and get as many governments as possible to sign formal protocol documents for their

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Edge of the Sahara: the bare sand is caused by grazing of livestock and cutting of trees for firewood.

implementation well in advance of UNCOD. As a result, when the conference was convened, there was considerable optimism about the seriousness with which they would be implemented. Since then, however, there have only been statements of good intention by political leaders. President Houari Boumedienne, for instance, had assured Dr Mostafa Tolba, Executive Director of UNEP that Algeria would press ahead with the North African green belt covering Algeria, Egypt, Libya, Morocco and Tunisia. But there are very few signs of the speed and urgency that is required. These five North African countries together lose a total of about 100,000 hectares of productive land to advancing deserts every year, which such a project could prevent.

Nepal has expressed an interest in joining India, Iran, Pakistan and Afghanistan in the south-west Asian desert-monitoring-project but the political unrest in Iran, where the project's headquarters were to be situated, and the sudden change of government in Afghanistan have delayed this project too.

The politically calm Sahelian region is, however, faring relatively better, with a growing institutional base for desert control. An Interstate Committee for Drought Control in the Sahel (CILSS) has been set up which involves senior Ministers, and it is receiving international assistance through the Club du Sahel. Regional scientific research is being undertaken by the

new Institut du Sahel situated in Bamako, Mali. The United Nations Sudano-Sahelian Office based in Ougadougou, Upper Volta, is being expanded to help all the countries in this region to undertake desert control projects such as those proposed at UNCOD.

The Institut du Sahel will hopefully become a model for regional co-operation in deserts-related research and training. Since UNCOD, UNEP has organised two training courses for scientists and officials from developing countries, one on sand dune stabilisation in China and another on soil salinity and improved management of pastures in the USSR. Considering the enormous social and ecological differences between different desert regions of the world, clearly it would be much better to set up regional centres for desertification training and demonstration, and organise such courses within the regions themselves.

But because of the difficulties in organising effective regional co-operation in combating desertification outside the Sahelian region, Professor Kassas believes that stress should now be placed on encouraging national anti-desert programmes. These programmes could be integrated with national land and water management schemes which aim to raise and stabilise the productivity of the arid regions. National planners, who have to make the best of scarce national resources, are more likely to be attracted by rational desert development

schemes, rather than those which are seen as simply desert control schemes or desert monitoring exercises.

The problem of growing deserts is indeed urgent. In the early stages of desertification, the cost of reclaiming land is relatively modest, but if neglected, the cost climbs steeply, until reclamation becomes economically impractical. Global land-loss from man-made causes is already estimated to cover an area bigger than Brazil. Estimates presented at UNCOD revealed that more than 680 million people are living on land that can no longer support permanent agriculture, or is showing signs of steady deterioration. These lands today produce a very large proportion of the global production of cereals, fibres and meat. It is therefore vital to restore them back to health.

Desertification issues will be raised at yet another international conference in Rome in July, 1979: the World Conference on Agrarian Reform and Rural Development (WCARRD), being organised by the Food and Agriculture Organisation. The latest WCARRD newsletter states: "There is no technology problem. Answers lie in mobilisation of national and international resources and popular participation in anti-desertification programmes. These issues will be on the WCARRD agenda." It can only be hoped that WCARRD will succeed where UNCOD has failed. □

Anil Agarwal is assistant director (editorial) of Earthscan.

African chemists hit back at foreign technology

THE conflict of interest between 'foreign' companies in developing countries and their hosts "can, and does, have a significant adverse effect on the growth of industrialisation in many developing countries and is at least one of the major causes of the slow pace of the development of industries in these countries", according to Professor D. E. U. Ekong, Vice-Chancellor of the University of Port Harcourt, Nigeria. And he is not alone in his criticisms. At a recent UNESCO symposium on university-industry interactions in chemistry, all the African delegates agreed that the system of itinerant expatriate advice is totally irrelevant to African needs.

In Nigeria, said Ekong, the law now requires majority Nigerian shareholding in most enterprises as well as significant Nigerian representation in management. Nevertheless, technical management is still dominated by the foreign technical partners who determine the technology and the process, including the raw materials, for the in-

dustry. Thus the foreign technical partner plays a crucial role in determining the course of industrial development.

Ekong accused these partners of giving biased advice. Their interests as manufacturers, or even their desire to dispose of equipment obsolete in their own countries, will affect their recommendations. Manufacturing processes that require raw materials or key intermediates from the parent company are often chosen.

The Nigerian desire to reduce their dependence on imported machinery and raw materials is thwarted by the lack of provision for research and development. The foreign technical partner provides all the technical know-how and solves any technical problems. Even where the manufacturing process requires quality control involving chemical or physical analysis, many firms use unqualified foreign technicians. Hence, says Ekong, "chemists in developing countries have very few opportunities".

According to A. O. Shobo, of Lever Brothers, Nigeria, however, the situation is slowly changing. While it used to be the case that all R & D problems were referred to headquarters for solution, there is now a gradual recognition that there is a limit to the scope of R & D that can be carried out without an adequate knowledge of local conditions. Local companies are gradually setting up their own R & D activities.

The majority of scientists in Nigeria are in the universities. They have a major role to play in developing local industry based on local raw materials and appropriate technology. But they face many difficulties, the main one being financial. To study a problem until the results are applied in industry requires continual and intimate collaboration between industry and the university which few local enterprises can afford. It is easier to buy imported processes even where this means importing preprocessed raw materials or intermediates.

Ekong suggested solving this problem by compelling all industrial enterprises in Nigeria to contribute to a fund for local industrial R & D. They would be able to claim rebates depending on how much they spent on local R & D. He also suggested that tax incentives be given to industries based on local knowledge and raw materials.

Ekong believes that without significant input from the universities there

will be no self-sustained growth in industrialisation in the foreseeable future. However, because governments regard research facilities as luxuries developing countries can ill afford, "most chemical laboratories in our universities are ill-equipped and lack basic facilities for even the most elementary research", says Ekong. "Lack of scientific leadership is the principal reason for the low scientific

output and is an area where international collaboration could be fruitful. Dr C. C. Mjojo from Chancellor College, Malawi, pointed out that there was little difficulty in finding money for applied research but support was needed for the pure natural sciences.

Lynette Hamblin

Lynette Hamblin is editor of Education in Chemistry



Pakistan reorganises agricultural research after harvest disaster

AFTER a disastrous wheat harvest in Pakistan last year (the shortfall in expected production was 1 million tons), the Pakistani government has had to import 2.2 million tons of wheat at a cost of 4,000 million Rupees (\$400 million). The bad crop came as a particularly hard blow because the country had hoped to achieve self-sufficiency in food last year and wheat is its staple food.

The poor crop can be attributed to the fact that 75% of the wheat was afflicted by rust. The main reason for such widespread infection seems to be due to the fact that the popular commercial wheat varieties used had not been bred for rust resistance.

The Pakistani government lays the blame with its agricultural policy and the way in which the country goes about agricultural research. There are agricultural research institutes in Pakistan but most of them are isolated in the provinces and suffer from a lack of central coordination. Most of their research on breeding has centred on improving yields. Breeding resistance to adverse conditions such as disease or weather has been largely overlooked. And many very important aspects of agricultural research such as climatology, plant biology, agronomy and pathology are barely covered at all.

Dr Amir Mohammad, chairman of the Agricultural Research Council in Karachi has plans to improve Pakistan's agricultural research. The ARC has already drawn up the following measures for quick implementation:

- The creation of a National Agricultural Research Centre. This is already being built in Islamabad on 1,300 acres of land. It is planned as a centre of excellence for agricultural research and is being funded by Pakistan and to the tune of \$19 million by foreign grants and loans.

The centre will consist of institutes on agricultural climatology, plant biology, agricultural data and planning, agricultural engineering and machinery, forestry, fisheries, water-shed management, arid-zone research, rural sociology, and a national plan introduction centre. Building should start in mid-1979 and the centre should be functioning in about three years time.

- The creation of a National Commodity Institute under the ARC. This will conduct research into individual crops and livestock. It will consist of separate sections for each major crop, such as wheat, rice, sugarcane, and cotton and a livestock section. Existing provincial research institutes will remain but will be under the NCI and the NARC.

- Manning of the institutes. 150 first-rate scientists will be needed. Pakistan is short of such manpower, so it plans to put new graduates into a six-month training course and then to send most of them for further training abroad.

- The division of the country into eight agro-ecological zones each carrying a different climate and ecology. An advisory committee of scientists and local farmers for each particular zone, will meet periodically to assess local



Grain production in Pakistan. Top left: terraced farming. Above: grinding wheat.

conditions and recommend measures for ensuring optimum output.

- Strengthening of the small cereal Diseases Research Institute operating under the ARC so that it can incorporate studies of new cereal pathogens and screen new cereal strains for resistance to various rusts.

- The development of a coordinated research programme. ARC plans to have a strong research group on the socio-economic aspects of agriculture, which will also deal with large and small-scale planning of the country's agricultural resources.

At a Wheat Research and Production Seminar held in Islamabad last August, Dr Norman Borlaug, Director of CIMMYT, Mexico, expressed the view that Pakistan could increase its wheat production from 8-9 million tons to 15 million tons if farm production was made more efficient. At present, in spite of the 'Green Revolution', the wheat yield in Pakistan is quite low. As against Mexico's 4,175 kg/Hectare, Egypt's 3,343, and the US's 2,022, wheat production in Pakistan is only 1,411 kg/Hectare.

Azim Kidwai

correspondence

The Piltdown hoax

SIR,—Halstead's preliminary presentation of allegedly new material on the Piltdown hoax¹ could not reasonably be expected to encompass all the evidence which has accumulated in relation to anthropology's most notorious forgery. Nonetheless, one might have hoped that the tape-recorded allegations of the late Professor Douglas would have been accorded a tolerably critical analysis. Such is not the case.

Douglas and Halstead's attempt to implicate Sollas (and Dawson), whilst it may appear plausible to the casual reader, at best represents only a small part of the truth about Piltdown, and at worst depends upon exaggerations and imprecisions. Let me give some examples.

Although Halstead does not qualify Douglas' assertions about Dawson's reputation, they are in fact grossly misleading. Whether or not Dawson was guilty of Piltdown, he certainly did not acquire a reputation for perpetrating hoaxes during his lifetime. In fact he was a respected collector of fossils and antiquities for the British Museum. To be sure, after the exposure of the Piltdown forgery in 1953, a number of insinuations against Dawson's integrity were assembled,² and it has recently been shown that some Roman brick-stamps which Dawson donated to the British and Lewes Museums are forgeries.³ However in the first instance the insinuations may represent retrospective mud-slinging by local Sussex antiquaries jealous of the international fame which Dawson achieved through Piltdown, and in the second instance the possibility remains that Dawson was the dupe rather than the forger. Certainly prior to his death in 1916, there was not a hint of scandal about Dawson. Even in relation to Piltdown itself, Dawson's guilt is far from proven. Of the three full-length books written about Piltdown after 1953, two argue that Dawson was innocent,⁴ and the third, while implying Dawson's probable guilt, pointedly avoids expressing its final conclusion in positive form.⁵

Apart from glaring inaccuracies such as the above, the Douglas/Halstead hypothesis embodies many statements which are so vague as to be practically worthless as evidence. The potentially crucial assertions that Sollas knew and visited Dawson, that Sollas received a packet of potassium bichromate, and Sollas borrowed some ape's teeth, are left with no firm indications as to when these events allegedly occurred. If they took place after December 1912, their occurrence would almost certainly be irrelevant to the hoax. Even the incident of the Sherborne Horse's Head, which constitutes the most suggestive part of the Douglas/Halstead case, is open to many interpretations.

Certainly a Sollas/Dawson conspiracy would provide a possible, perhaps even

a likely, explanation for the Piltdown hoax, but the best evidence for such a conspiracy lies in areas untrod by Douglas and Halstead. The 1911 edition of Sollas' *Ancient Hunters* provides a detailed description of "Heidelberg man", an earlier candidate for the title of "missing link" which probably served as a model for Piltdown, and which is known to have preoccupied Dawson circa 1912. Moreover, since the Piltdown forger chemically treated the cranial fragments in an attempt to make them appear fossilised,⁶ it is surely worth noting that Sollas had done important pioneering work on the chemistry of fossilisation,⁷ work which Dawson may well have utilised in interpreting the Piltdown remains.⁸

However, because of problems surrounding Sollas' motivations, problems which Halstead's communication fails to resolve, I am inclined to lay the blame for Piltdown elsewhere. My current working hypothesis is that Elliot Smith instigated the hoax, and that Smith Woodward may have been a willing accomplice. This hypothesis does much to illuminate a formerly puzzling episode from Australian prehistory, an episode in which, incidentally, Sollas played a supporting role.⁹

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Allies against Lysenko

SIR,—In Vera Rich's moving account of Professor Lev Tumerman's seven years in solitary confinement (14 December, page 662), she mentions that he recalls "one very good book by Sunnot and Dent". He no doubt meant to refer to the classical textbook of genetics by Sinnott, E. W. and Dunn, L. C. *Principles of Genetics*, McGraw-Hill, New York and London, 1925 which went through several editions (the last with Dobzhansky) and which for almost four decades (1925–1965) was the standard text in this field. E. W. Sinnott was professor of botany at Columbia and then Yale and L. C. Dunn was professor of zoology at Columbia. Dunn, who died in 1974, was active in the field of developmental genetics for more than a half-century; a fascinating review of his life and work has appeared recently (Bennett, D. *Ann. Rev. Genet.* **11**, 1–12; 1977).

It is interesting that Dunn, who was often bitterly attacked in the United States for his devotion to what were at

that time left-wing causes, was an outspoken critic of Lysenko and of the Soviet persecution of their geneticists and of other scientists and intellectuals, such as Tumerman himself.

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Who assesses risk?

SIR,—I was much struck with Wendy Barnaby's report of the Swedish seminar on risk assessment (7 December, page 554). The impression conveyed was that informed and impartial politicians ought to regard a democratic society's evaluation of risk perception as even more important than quantified objective risk. This is tantamount to saying that it is more important to consider the number of people who would be frightened than the number of people who would be killed.

The conclusion is that one should favour energy production from fossil fuels which kill far more people, rather than nuclear power which frightens far more people.

I can see why short-sighted politicians might approve this view, assuming that the fears of the public will last at least until the next election. Dead people don't have votes. Frightened people do. But I am surprised that *Nature* appears to approve the same opinion.

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Not by force of ARMS

SIR,—I welcome the article by Joe Schwartz (23 November, page 310), drawing attention to the unsatisfactory job security and career prospects of research staff. Technicians and graduate research assistants, as well as post-docs, are often victims of short term contracts.

May I underline the point that it is the quality and strength of local trade union organisation which determines how effectively these issues can be negotiated with each university, medical school, college or institute. For this reason I was alarmed by the suggestion that ARMS "intends to negotiate with employers". Indeed, I am sure it would delight these employers to be relieved of the embarrassing task of trying to justify their practices to the experienced negotiators of strong trade union organisations. I welcome ARMS to the extent that its existence demonstrates the concern felt by research staff, but I must qualify this by warning that the ARMS could backfire if it diverts staff from the need to work within trade unions.

Finally, I should clarify that I am not a "division organiser" for ASTMS (as reported in your article). I am one of many lay activists within ASTMS who have been tackling these issues, and many others, for some years.

PHILIP HOUNSELL

London, UK.

news and views

Quaternary correlations

from D. Q. Bowen

FOR the foreseeable future the major challenge confronting Quaternary research must be correlation of the oceanic and continental records. Continental models of Quaternary history are now inadequate and already a re-evaluation has commenced. Astonishingly this has been long delayed, for the implication of such inadequacy, and of greater complexity was plain for all to see from Emiliani's work on stable isotopes from deep sea cores during the early 1950s. Partly the delay was due to misconceived interpretation of the oceanic evidence in the light of continental models, whereas now, despite some lingering opposition, the horse has at last been placed firmly in front of the cart.

Notwithstanding the eminently reasonable geological suspicion of notions of continuous sedimentation, the open ocean (pelagic) environment constitutes a special case. There sedimentation by settling through the ocean takes place more or less constantly for long periods as demonstrated by inter-core correlation throughout the world's oceans based on repetitive isotopic signature, palaeomagnetic character, assemblage faunal analysis and, increasingly, by the extinction of certain taxons at isochronous levels.

The oceanic oxygen isotope stratigraphic framework shows the changing isotopic composition of the world's oceans throughout the Quaternary. Preferential evaporation of the lighter ^{16}O and its incorporation into ice sheets on the continents tends to enrich the oceans residually in ^{18}O during glacials. Conversely during interglacials isotopically lighter water is returned to the oceans. Foraminifera from deep sea cores, which precipitated their calcareous tests in isotopic equilibrium with the water they inhabited, monitor such changes, and allow definition of oxygen isotope stages numbered backwards in time, using odd numbers for interglacials and even ones for glacials.

This stratigraphic framework records changing ice volumes on the continents, and by inference the changing climate also. It shows that during the 1.6 Ma of the Quaternary some 18 glacials occurred which compares with the celebrated four-fold glaciation model of the Alps. This has dominated thinking for so long although it only represents the last 850 ka during which time 10 actual glacials occurred. The extent to which the Alpine model influenced others and the terms on which new data were collected and evaluated in terms of a four-fold sequence, is only now becoming apparent with the realisation that much of the primary data base in terms of basic field geology is inadequately known in many areas.

Without geochronometric control, for example ^{14}C , uranium series and K/Ar, traditional means of correlation continue by plotting data graphically to show fluctuations of climate through time. Correlation is principally by telecorrelation relying on curve-matching assisted occasionally by radiometric tags at crucial points. Hitherto the best results, which bear an amazing resemblance to the oceanic record, come from the loess terrains of central Europe (with signs that work on the loess of China is not far behind). There, sequences of loess, palaeosols and other indicator deposits, coupled with the recognition of palaeomagnetic reversals for dating, allow correlation back to the beginning of the Pleistocene. Other key localities include the long lake sequences of the Vosges, Macedonia, Colombia, Queensland and Japan, where climatic variations are

recognised from pollen analysis. Such a technique is eminently successful when applied to continuous layers of sediment, but rather less so when the data are fragmentary and stratigraphic succession inferred by an additive process using geographically widespread data. One pollen sequence may look much like another for the fact is that repeated constellations of species reappeared during successive similar climatic episodes, perhaps with different emphasis in the relative timing of arrivals, but as the regional variation of the vegetation is largely unknown this cannot be used for definitive dating. For Northern Europe it was formerly thought that the interglacials of the Middle and Upper Pleistocene were known and sufficiently well differentiated—Cromerian, Holsteinian and Eemian. However, in the light of the oceanic evidence, there must be at least six interglacials. Clearly the problems are beyond the resolving power of the method, which is not surprising since it relies not on first appearances nor extinctions, but on assemblage floras, a biofacies basis which is inherently unreliable on account of its diachronous nature.

Using this method marine deposits from Western Norway are correlated with the Eemian Interglacial and also with oxygen isotope stage 5e (Mangerud *et al.*, this issue of *Nature* page 189). In equating Eemian with stage 5e the authors follow Shackleton (*Proc. Roy. Soc. Lond. B.174*, 135; 1969) and there can be little doubt that the Last Interglacial at 125 ka is a prominent feature of sequences throughout the world. The chrono-

Oxygen isotope stage	Classical continental stages
1	'Holocene' (postglacial)
2	Weichselian (last glaciation)
3	
4	
5a-5d	
5e	'Eemian' (of Schleswig-Holstein)
6	Warthe
7	'Eemian' (northern Germany)
8	Reburgh
9	'Eemian' (Amersfoort)
10	Saale ss

Eemian sl
Saale sl

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stratigraphic term Eemian, however, is potentially misleading, for Kukla makes a compelling case for the recognition of three stratigraphically separate Eemian Interglacials (*Earth Sci. Rev.* 13, 307; 1977). The type Eemian, considered by the authors, is near Amersfoort in the northern Netherlands. It lies between Saale glacial deposits and sands of the Last (Weichselian) Glaciation (isotope stages 2, 3, 4 and 5a-5d). Kukla argues that three glaciations occurred during the period traditionally referred to as Saalian (Saale(ss), Rehburg and Warthe), and that the Saale(ss) corresponds to oxygen isotope stage 10. Thus because the Amersfoort Eemian deposits appear to be continuous with the underlying Saale(ss) they should be correlated with stage 9. Eemian of isotope stage 5e is probably represented by the interglacial deposits of Schleswig-Holstein where marine beds clearly overlie and breach end-moraines of the Warthe Glaciation (isotope stage 6). Clearer stratigraphic demonstration of two Eemian Interglacials (probably corresponding to stages 5e and 7) is found in Northern Germany where they are separated by glacial deposits (Wiegank, *Geologie (Berlin)* 21, Beih. 77, 1; 1972).

Correlation of the Norwegian deposits with the type Eemian (stage 9?), on floristic grounds, and also with stage 5e, is, therefore, not unequivocal. It may be that correlation with the Eemian of Schleswig-Holstein (5e?), is sounder, because faunal evidence from the Norwegian deposits, showing higher temperatures than today, matches stage 5e from deep sea cores in the Norwegian Sea. Such a correlation is not as tenuous as might appear for comparable temperatures have only been recorded twice in the last 450 ka.

The problem of 'Eemian', and other similar terms, remains, and is symptomatic of the state of continental frameworks at the present time. These require urgent revision and redefinition in the way Kukla has attempted for Europe. It may mean a plethora of new stage terminology, often on a local if temporary basis, but until the basic ground is secured in this way progress will be hindered. The existing continental frameworks cannot continue to bear the brunt of attempted correlation with oceanic or other records either for the specialist or numerous users of Quaternary data. □

denses and is then surrounded by protein.

Several of the 50 papers presented in Toronto dealt with the question of size determination. That is, how is it that virus substructures such as heads and tails are virtually always the right size, even though their component proteins can frequently be shown *in vitro* or in mutants to be capable of forming structures of a variety of different sizes? An interesting case is found in *Escherichia coli* phages P2 and P4. P2 by itself makes uniform sized heads. However, if a cell is co-infected with P2 and P4 (a satellite phage which itself codes for no head protein) then the P2 head proteins are assembled into smaller P4-sized heads. Assembly of P2 proteins into P4 heads is directed by a P4 gene that has been named *sid* (size direction). J. Geisselsoder, M. Chidambaram, and R. Goldstein (Harvard University) presented evidence that the *sid* gene influences the efficiency of transcription termination on the P2 genome. They proposed that in the presence of the *sid*⁺ gene product the relative amounts of certain P2 head proteins are altered, and that it is these crucial ratios that determine the size of the head assembled from the head proteins.

In the case of size determination of T4 heads, interest centred on the well known fact that the head is elongated in one direction. Genetic studies reported by D. Doherty (University of Washington) show that mutations in the gene coding for the major head protein (gp23) which cause the production of non-elongated heads can be suppressed by simultaneous mutations in genes 22 and 24. These genes code for the protein (gp22) of the scaffold around which gp23 assembles and the protein (gp24) that constitutes the corners of the head. The results suggest that the three proteins interact with each other to determine head length. L. Onorato and M. Showe (University of Basel) presented biochemical experiments which also implicate these three proteins in head length determination. Their experiments further suggest that the ratios among the concentrations of these proteins influence the size at which the growing shell is capped off; thus they arrive at a conclusion analogous to the results with P2 and P4 heads by a different experimental route. R. van Driel (University of Basel) described reconstitution experiments with purified proteins which showed that the scaffold protein (gp22) will assemble in the presence of an initiator protein (gp20) into what appear to be normal

Bacteriophage assembly

from Roger W. Hendrix

ANY recent textbook of cell biology or biochemistry contains a diagram based on the experiments of Edgar and Wood that outlines the assembly of bacteriophage T4. In the best tradition of Henry Ford, protein and DNA molecules assemble sequentially into the component parts (heads, tails, tail fibres), and these subassemblies then join each other to form the finished product, the virion. This scheme of phage assembly has been incorporated into biochemical lore, widely quoted, sometimes overinterpreted, and not infrequently taken to represent a relatively complete understanding of virus assembly. However, despite the elegance and importance of Edgar and Wood's original experiments, basic questions are still unanswered, and phage assembly remains a fertile field for investigation into the principles of biological form determination. Some of the highlights of recent work in the field were aired last September at the Sixth Biennial Conference on Bacteriophage Assembly.

Forming the intellectual backdrop to the meeting were a number of major conceptual advances in understanding virus assembly that have developed over the past decade. Some of these are the following. Assembly pathways

are in general quite strictly ordered and, although all of the relevant proteins are present together in the cell, they associate with each other only when the pathway requires it. In other words, the order of assembly is regulated by the assembly pathway itself rather than, for example, by the order of transcription or translation. Virus assembly, at least in the case of the structurally elaborate phages, is more than a simple 'self-assembly' process in which all of the information for assembly resides in the components present in the final assembled product. Although many individual steps in the assembly of these viruses undoubtedly involve true self assembly, other steps in the process require the intervention of proteins that act as assembly tools or catalysts and are not themselves found in the completed virion. Among the 'tools' that are beginning to be characterised are proteins that form transient scaffolds which direct the assembly of other proteins, and enzymes that cause covalent alterations of proteins already assembled. The actual DNA packaging event has been a central issue for many years, and it has become clear that DNA is condensed into a preformed protein shell—this in contrast to the original idea that the DNA first con-

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sized scaffolds. This argues that at some level the scaffold protein 'knows' the correct head length even in the absence of gp23 and gp24.

The mechanism of DNA packaging has been a favourite problem in the field, and there has been impressive progress in recent years, particularly since it has become possible to package DNA *in vitro*. The papers on DNA packaging in Toronto ranged over a wide variety of topics including how the DNA is recognised by phage proteins, how it is cut to its mature form, and the relation between cutting and packaging. One particularly interesting report for this field which has primarily been concerned with packaging double-stranded DNA, was a paper by K. Kothe and D. Dressler of Harvard University on packaging the single-stranded DNA of ϕ X174. Their evidence shows that the preformed protein capsid is associated with the replicating DNA, apparently as part of a complex at the replication fork of a rolling circle, where it takes up the DNA as it is made. Replication and packaging of DNA are tightly coupled in this case, and it is tempting to speculate that the driving force for packaging might be derived from replication. This is in marked contrast to the case of double-stranded phages where replication and packaging are clearly separable.

Two models were presented for how DNA is moved into the heads of double-stranded DNA phages (L. Black, University of Maryland; R. Hendrix, University of Pittsburgh). These, if nothing else, reflect the current state of experimental evidence on the problem; they both differ from previous models in postulating that the force that drives the DNA into the head is applied by a small proteinaceous machine that sits in the head shell at the point where DNA enters (thought to be the point at which the tail later attaches).

The most stimulating paper at the meeting, to my taste, was one by J. Shaw and H. Murialdo (University of Toronto) which admits phage lambda to the club of viruses with overlapping genes. They find that the lambda head gene *Nu3* is contained within the last one-third of the *C* gene. The two corresponding proteins, gpNu3 and gpC, are read in the same phase and presumably use the same translational termination signal (that is gpNu3 is identical to the C-terminal one-third of gpC). The real interest in these results probably lies not in the fact that they give yet another example of overlapping genes but in the fact that it may be relatively easy to decipher the functional significance of the overlap. gpC is most probably part of the head initiation complex on to which the

scaffold protein gpNu3 assembles, and it is easy to imagine models in which the C-terminal portion of gpC might serve, for example, to nucleate the assembly of the gpNu3 scaffold.

Much of the research in phage assembly in past years has dealt with working out pathways, measuring compositions of intermediate structures, and otherwise defining the outlines of assembly processes. It was evident in Toronto that the emphasis is now moving toward more detailed mechanistic questions at the level of how proteins and parts of proteins interact with each other and with nucleic acids. For example, two elegant structural studies addressed the question of how the protein subunits of the head shell change their conformation as the shell expands during DNA entry. S. Casjens (University of Utah) used freeze-etch electron microscopy to argue that the major head shell protein, gp5, of P22 consists of two domains and proposed that head expansion could be accomplished by the flexing of a hinge connecting the two. A group from the University of Basel (U. Aebi, R. van der Broek, P. R. Smith, B. ten

Heggeler, J. Dubochet, V. Mesyanzhinov) examined the analogous lattice expansion in T4. By using electron microscopic image processing techniques and antibody fragments, they were able to visualise a specific antigenic site on the T4 major head protein gp23. They found, surprisingly, that on lattice expansion this site moves from the inside to the outside surface of the head.

The Toronto meeting was noteworthy for the consistently high quality of the papers, most of which have necessarily been skipped over in this report. It was particularly gratifying to see that for a number of the interesting problems of phage assembly there is now a large body of information from each of several phages. This has made it possible to begin to decide which features of a given assembly process are fundamental and which are variations on a theme. The general impression was that the groundwork for understanding assembly of phages has been solidly laid and that future work will be progressively more concerned with problems that can be generalised to other biological systems.

Muons and muon trapping by solid-state imperfections

from A. M. Stoneham

THE positive muon has an obvious potential in solid state physics. Its spin polarisation can be monitored to give data such as the site it occupies, local magnetic fields, diffusion rates and Knight shifts. If a suitable beam is available from a meson 'factory', the experiments themselves do not need especially sophisticated equipment. So it is not surprising that the μ SR field is very active (see *News & Views* 276, 324; 1978) both in an attempt to understand the way muons behave in solids and to exploit muons to understand better the solids in which they decay.

Several groups have found evidence that muons can probe defect and impurity properties in metals. Since the defects include deformation-induced dislocations and damage from neutron irradiation, and since the impurities include both alloying metals and trace impurities like oxygen, the muon promises to be a very versatile tool. Many gaps remain. To understand these, one must look at the principal processes involved. The first is the slowing-down process in which the muon comes to rest. Usually the muon

will end in an interstitial site, self-trapped by the lattice distortion it causes, and vibrating like any normal chemical impurity. The second process is diffusion. Presumably this is described by the quantum theory developed for light interstitials by Flynn and Stoneham; the features of importance here are that the rate should increase with temperature, as a power law at low temperatures and thermally-activated at higher temperatures. One might expect μ^+ diffusion to resemble diffusion of H^+ , D^+ or T^+ , with a suitable isotope effect, though present data are too limited to check in detail. The third process is defect trapping, due to the strain fields of imperfections or to their perturbation of the electronic structure. At high temperatures, release from traps occurs. There is also a fourth process, independent of the solid: the muon decays with a characteristic time of 2.2 μ s, and this imposes limits.

If there is only a single type of trap, one can outline the way the depolarisation rate should vary with temperature. Four main regimes are expected. In regime I, at the lowest temperatures, the muon does not diffuse significantly; it merely comes to rest, and measures an average property of the solid. In II

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One of those curious apparent imperfections of nature is the lopsidedness of electricity and magnetism. The world abounds with electric particles (all atoms are made of them) but nobody has ever seen a pure magnetic particle. Magnetic fields can, it seems, only be produced by moving electricity, that is, electric currents. Consequently, when magnetism occurs, magnetic charge always appears in tandem (north and south), never singly. Tandem charges are called dipoles and single charges, if they exist, are known as monopoles. Matter is built out of subatomic electric monopoles, but magnetic monopoles elude us. This is all the more surprising because of the celebrated mathematical symmetry built into our theory of electromagnetism.

The great nineteenth century physicist James Clerk Maxwell unified electric and magnetic fields, and discovered the principles which govern their interplay, but his beautiful theory is marred by the asymmetry of the monopoles. In the 1930s Paul Dirac gave an argument, subsequently developed by Julian Schwinger, that if magnetic monopoles exist they will have magnetic charge only in fixed multiples of a fundamental unit, simply related to the fundamental unit of electric charge, such as we find on the electron. A few years ago the Soviet and Dutch physicists A. M. Polyakov and G. 't Hooft argued that magnetic monopoles could exist on subatomic particles with masses in excess of five thousand proton masses.

There has always been room in theory for magnetic monopoles, but elaborate searches among ordinary matter have persistently failed to detect any. Despite a recent flurry of excitement that one had been found in cosmic ray debris, the monopole remains perhaps the most significant non-discovery of the century.

Sometimes negative evidence can be useful in revealing information about other physical systems. The non-detection of quarks, for example,

Primaeval magnetic monopoles

from P. C. W. Davies

has been used as a motivation for theories of subatomic particle structure. In a recent paper (*Phys. Lett.* **793**, 239; 1978) the Soviet mathematical physicist and astronomer Ya. B. Zeldovich, and a colleague at the Institute of Applied Mathematics in Moscow, M. Yu. Khlopov, have argued that the experimental absence of magnetic monopoles raises serious doubts either about their existence or the popular understanding of the origin of the Universe. The central idea is the theory that the Universe began with an intensely hot big bang. Indeed, so energetic was this event that we can be sure that any subatomic particles that can exist would have existed at sufficiently early moments (that is, sufficiently high temperatures). And this includes magnetic monopoles, despite their predicted enormous masses.

Zeldovich and Khlopov calculate that inside the first 2×10^{-11} s the monopoles would have been in thermodynamic equilibrium at a certain concentration among all the other matter, but that as the explosive expansion rapidly cooled the primaeval cosmological material, the temperature fell too low to sustain the monopoles, and they started to annihilate each other. The annihilation is a consequence of fundamental physics that a magnetic north pole is the antimatter image of a south pole, and close encounters between them result in mutual destruction.

In the subsequent phases of the expanding fireball, most of the monopoles would have disappeared as a result of this mechanism. But not all. Merely on statistical grounds some monopoles would have avoided their opposite numbers and escaped this demise. This is because both the density of matter, hence monopoles, was

falling rapidly, and the temperature, hence speed, of subatomic particles also plummeted. The authors calculate the residue of relic monopoles coughed out of the big bang which should still exist. The annihilation ceases after about 10 ms, leaving a total concentration today of around $4 \times 10^{-18} \text{ cm}^{-3}$, or less than one monopole per million million atoms. Small though this is, it is enormously greater than the experimental upper limits already set ($10^{-30} - 10^{-38} \text{ cm}^{-3}$) from examination of lunar and terrestrial matter, and cosmic ray studies.

There seem to be two alternative explanations of this inconsistency. One is that nature truly is lopsided, and that magnetic monopoles simply do not exist in isolation. (There is still the possibility that for some obscure reason monopoles exist in permanently confined multiplets, like quarks.) The second is that the standard hot big bang model of the Universe is badly wrong, at least in some of its details. We thus have an additional motivation for seeking out monopoles, for their discovery as free particles would, unless the previous abundance estimates are a fluke, tell us much about the first micromicrosecond of the Universe.

One of the ironies about papers of this type concerns the central role played by aesthetics. Belief in magnetic monopoles stems primarily from a deep-rooted desire that nature should be elegant and symmetrical. Similarly in cosmology, sweeping assumptions are made about the structure of the primaeval Universe which, once again, stem from the yearning for simplicity. Now we see that cosmic simplicity (primaeval thermodynamic equilibrium) and subatomic symmetry are in conflict. The resolution cannot be found by aesthetics alone. Only experiment can decide which assumption is wrong.

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the muon diffuses faster and faster as the temperature rises, and the rate at which it samples different internal magnetic fields affects the depolarisation rate and can be measured. This regime ends at a temperature fixed by the trap density, and in region III, the muon monitors properties of the trapping site. Finally, in region IV, release from traps and motional-narrowing occur. Obviously, behaviour gets more complicated when several distinct traps are involved, but the principles are the

same. Many workers have obtained results fitting this qualitative description of the temperature dependence.

For this picture to be useful, it is important that the muon can indeed diffuse to the traps of interest. The muon lifetime sets a limit here. In most metals studied (V, Nb, Ta, Cu, Fe) at accessible temperatures the muon hopping time between diffusive jumps is in the range 10^{-3} – 10^{-7} s corresponding to a mere 0.2 to 20 jumps in a lifetime. Diffusion from a random stopping site

to any trap present in low concentration is negligible. And indeed the irradiation of Cu and the doping of Nb by Ta have little observable effect on muon properties. Two metals are different, and appear to show very fast diffusion: Cr, with a hopping time around 10^{-11} s and Al, where motional narrowing appears to be complete even at the lowest temperatures, with times such as 10^{-10} s at 10 K and 10^{-12} s at 50 K indicated.

Several groups have studied trapping

in Al, including workers at laboratories in America, Sweden and Germany. Two results are particularly interesting. One was reported by Seeger at a recent Rutherford Laboratory meeting, and is from Stuttgart workers, who looked at neutron-irradiated Al. The point here is that the basic defects, and hence the traps, can be modified by annealing. Reversible changes with temperature reflect the muon motion, whereas irreversible ones indicate changes in defect structure and can be related to previous damage work. Two traps were detected in Al: one was shallow, possibly an interstitial cluster; the other trap was deeper and is not yet identified. The second result comes from a collaboration among five of the American laboratories (*Phys. Rev. Lett.* **41**, 1558; 1978). The experiments showed the effects of Cu impurity and of deformation. The dislocations act as deep traps, with a binding of around 0.2 eV, and also seem to show the rapid pipe diffusion known from conventional diffusion studies. The Cu appears to give rise to some four different shallow traps (binding the muon by less than 1/50 eV) even at the low concentrations used (420 and 1,300 p.p.m.). The authors argue these traps are microclusters of Cu, and that the clusters are present at levels far above random. If so, the muon technique is giving new information about alloy structure in circumstances hard to match otherwise.

What then are the prospects? If the interpretations are right, muon spin resonance adds another tool to the study of alloy structure, complementary to positron annihilation, neutron scattering and various forms of electron microscopy. Clearly muon experiments need a large accelerator, and cannot be as portable as techniques like positron annihilation; applications to macroscopic defect structure, as in non-destructive testing, will have to be reserved for special cases. There are also some open questions. Are there really so many microclusters in Al:Cu? Are there systems where such muon results can be checked by other methods, like magnetic or Mössbauer techniques? If these microclusters are

real, do they affect mechanical properties? Another question is in how many systems can the muon probe defects and impurities? Diffusion rates mentioned earlier suggested that the success with Al was the exception, not the rule. This need not be so. Slow diffusion only prevents the muon probing a trap if the stopping site is random within the crystal. However, a slowly-moving muon may be stopped preferentially near defects, by its interaction with their strain field or otherwise. This may be why results on vanadium are

very sensitive to interstitial oxygen. In such cases the muon will probe the defects, but will give little information about the perfect host. Non-metals present their own special problems. Ionisation-enhanced diffusion in semiconductors may ensure the muon can always reach a defect; recombination-produced vacancies and interstitials in ionic crystals may ensure there are always defects to be reached. All in all, the exploitation of muons promises to yield scientific problems as well as solving materials ones. □

The salmon, the lungfish and the cow: a reply

THE debate on cladism has smouldered in specialist journals for over a decade, and we are glad that Halstead has brought it to *Nature* (*News & Views* **276**, 759; 1978). An adverse review (Cain *Nature* **216**, 412; 1967) of Hennig's book (*Phylogenetic Systematics* University of Illinois Press, 1966) is about the only previous sign of the controversy in this journal. As participants in the meeting at Reading, named in Halstead's report, we find his account seriously defective in two respects—as a description of the meeting, and as a summary of the claims of cladism and the history of the controversy.

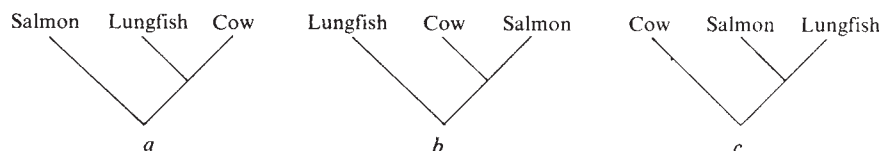
On cladism in general, Halstead's report reveals several assertions and misunderstandings about aims and methods that were common in the early days of the controversy, and have been fully discussed in *Systematic Zoology* and other journals. Rather than repeat those arguments, we should like to focus on what is, in our view, the principal source of acrimony between cladists and evolutionary systematists, a problem neatly summed up in a question raised at the Reading meeting and reported by Halstead: 'according to the cladists a lungfish is more closely related to a cow than to a salmon'. Halstead correctly reports the exasperation with which the accusation was put, and the complacency with which one of us accepted it. If experienced comparative anatomists can disagree on so simple and fundamental a question, either there is revolution in the air, or words are being used in different ways.

Given three taxa—for example, a lungfish, a salmon and a cow—a cladist will draw up the three cladograms showing the three possible 'more closely related' pairs.

Each of these concepts has its advocates, but at Reading, *a* was the preferred solution of the cladists, *us* included, and *c* was the preferred solution of Parrington and Halstead, whom we shall refer to as evolutionary systematists, following Mayr (*Z. Zool. Syst. Evolforsch.* **12**, 94; 1974). Since we do not believe that evolutionary taxonomists disagree with us over the characters of lungfish, salmon and cattle, the disagreement is evidently over the meaning of the concept 'relationship'. Although 'relationship', 'affinity' and cognate words have been freely used in the evolutionary and pre-evolutionary literature of biology, Hennig was the first, to our knowledge, to give an unambiguous definition of the concept. Mayr (*op. cit.*) summarised Hennig's definition of relationship; 'the measure of phylogenetic relationship is the relative recency of common ancestry'. Stung to give precision to a concept they had previously used in various senses, evolutionary systematists seem to have settled on 'genes in common' as the measure of phylogenetic relationship (Mayr (*op. cit.*) 'inferred amount of shared genotype'). Ignorant as we are of the correspondence between the genotype and phenotype, and of the genotype in the vast majority of organisms, 'genes in common' is a rationalisation of 'characters in common', or overall similarity. Hence, when evolutionary systematists prefer cladogram *c*, they mean that lungfish and

A hundred years ago

INVALIDS will be glad to learn that, amidst the severe weather, a comparatively mild climate exists in a part of their own country. A bouquet arrived from Glengarriff on Saturday, the 11th instant, comprising wallflower, primrose, primula, stocks, chrysanthemum, scarlet geranium, arbutus-berries, and a rose-bud, all picked from an exposed garden. In Madeira—but five days' journey—ripe bananas and custard-apples are hanging on the trees. Many of the gayest flowers are in full bloom. From *Nature* **19**, 16 January, 257; 1879



salmon are more similar than lungfish and cows. And in preferring cladogram *a*, cladists mean that they infer that lungfish and cows shared a more recent common ancestor than lungfish and salmon. This inference is drawn from the fact that lungfish and cows share derived characters (synapomorphies such as internal nostrils, an epiglottis, a two-chambered auricle and so on (Kesteven *Proc. Roy. Soc. Vict.* **59**, 93; 1951)) not found in salmon.

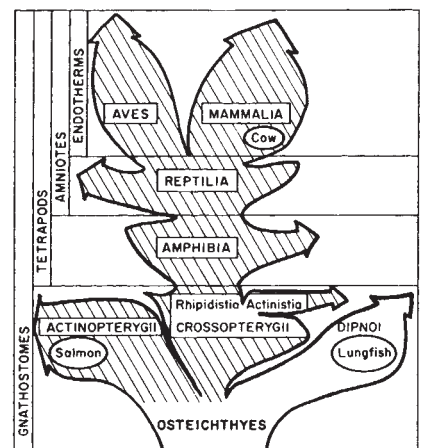
Given their reliance on overall similarity, it is not surprising that evolutionary systematists find that many of the groups they recognise are grades, nor that these grades 'happen to coincide with the major classes of the vertebrates' (Halstead *op. cit.*). Yet these vertebrate classes were recognised and named by pre-Darwinian systematists, who also used overall similarity as their guide, naturally enough. Overall similarity may be modernised or dignified by calling it 'genes in common' or 'shared genotype', yet when we do have access to comparative information on genes, as in globin sequences (Goodman *et al. Nature* **253**, 603; 1975; Romero-Herrera *et al. Nature* **261**, 162; 1976), the biochemists present their information in the form of cladograms, and use the same genealogical concept of relationship as Hennigians. Darwin (*Origin of Species*; see also Nelson *Syst. Zool.* **23**, 452; 1974) wrote 'our classifications will come to be, as far as they can be so made, genealogies; and will then truly give what may be called the plan of creation'. He might be surprised that after 120 years, some palaeontologists would be occupied with the defence of pre-Darwinian concepts and classifications.

Halstead's report contains many misunderstandings and ambiguities, a few of which cannot be allowed to pass unchallenged. It is not, as Halstead says of cladistics, "axiomatic that . . . sister groups are given identical ranks"; it is however a logical consequence if a formal classification is required. Nor does cladistics insist on a classification which reflects all of the ideas embodied in the cladogram. Cladists do not assemble characters subjectively into a hierarchy: though we suspect that the hierarchy of which Halstead speaks is a reference to character weighting, not favoured by cladists. Halstead also accuses cladists of belittling the significance of parallelism. But this is outside the scope of cladism since the recognition of such an evolutionary event presupposes that we already know the phylogenetic relationships of the organisms concerned.

L. B. HALSTEAD AND COLLEAGUES
REPLY: The mutual relations of salmon (an actinopterygian), lungfish (a dipnoan) and cow (a mammal, a class which can be traced back to rhipidistian crossopterygians by way of reptiles and amphibians) are certainly worthy of serious consideration. Gardiner *et al.* draw up "three cladograms showing three possible 'more closely related' pairs". Cladogram *a* is their preferred solution, but it is certainly not true to claim, as they do, that "c was the preferred solution of Parrington and Halstead". The cladists simply omit the possibility that none of the three pairings shared a more recent common ancestor, than any of the others, but rather were all derived from a pre-existing common stock, a view previously commended by Miles (*Palaeozoic Fishes*, 1971).

Assuming that cow, salmon and lungfish were derived from such a common ancestral stock, we would still insist that lungfish are closer to salmon on the grounds of neither having advanced significantly beyond the common heritage of all primary aquatic jawed vertebrates. In marked contrast, the cow is separated from both lungfish and salmon by several structural grades. The figure summarises these relationships.

At the meeting Gardiner presented a cladogram which purported to demonstrate that lungfish were more closely related to tetrapods than were either coelacanths or certain rhipidistian crossopterygians. The near structural identity of crossopterygians and early tetrapods makes it impossible for them to be separated by the uniquely specialised lungfish. Later in the meeting T. S. Westoll demolished this particular cladogram and pointed out that most of the features that Gardiner claimed were absent in coelacanths were in fact present in the material Westoll himself was studying.



similar pattern of skull roofing bones

Janvier's agnathan cladogram was a reflection of Stensiö's mistaken notion of the myxinoan affinities of the heterostracans, which has been discussed and firmly rejected by workers in the field (see *Biol. J. Linn. Soc.* **5**, 339-349, 1973; *Biol. Rev.* **48**, 279-332, 1973, for a summary of this issue).

The current Hennigian orthodoxy brings to mind the situation at the beginning of the present century. Then Haeckel's Biogenetic Law and the attendant preoccupation with genealogies, had a stultifying influence on the whole of biology. 'When the results of a science are produced by methodological assumptions rather than by the evidence available, it is not science' as A. J. Cain remarked in his review of Hennig's book in *Nature*.

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Concerning Halstead's report of the meeting itself, we were surprised to read that papers delivered by two of us 'were demolished within hours'. Freedom of speech goes a little too far here, and we request that Halstead justify his assertion by saying who did the demolition work, and how it was

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done. Halstead charges us with 'religious fervour', but the charge might be reversed, for to us his statement that cladists 'are already entrenched in some of the major museums in the world' has an offensive taint of McCarthyism and the witch-hunt.

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review article

Cellular interactions in haematopoiesis

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In vitro culture of haematopoietic cells has provided some surprising insights into critical interactions of blood-forming cells. Subpopulations of lymphoid cells have been shown to produce colony-stimulating activity, to interact with macrophages, and to have important effects on the very early stages of erythropoiesis. Macrophages have multiple influences on the proliferation and differentiation of other haematopoietic cells.

PRIMITIVE pluripotent haematopoietic stem cells have the capacity to form all the blood elements, including myeloid cells, erythrocytes, and immunocytes¹. In mice the stem cell for erythroid and myeloid lines is assayed by its ability to form haematopoietic spleen colonies in irradiated syngeneic recipients (hence, the stem cell is often called CFU-S, colony forming unit-spleen). The derivation of colonies from a single precursor cell was established by using a mouse with chromosome abnormalities as the cell donor. The same abnormalities were found in replicating cells of lymphoid tissues diffusely distributed in the spleens and lymph nodes of the recipient mice. Further evidence was derived by retransplantation of non-immunocompetent spleen colonies. Colonies developing in secondary host animals contained immunoglobulin-producing cells with the T6 chromosome marker of the original bone marrow donor. Direct evidence for a single stem cell common to the myeloid and lymphoid lines has recently come from reconstitution experiments in mice in which chromosome markers were induced in donor bone marrow cells by irradiation². Myeloid spleen colonies and T and B lymphocytes in reconstituted animals had the chromosome marker of a common precursor. The appearance of the Philadelphia chromosome in cells of the granulocytic, mononuclear phagocytic, erythroid, and megakaryocytic lines of patients with chronic granulocytic leukaemia is evidence for the existence of pluripotent stem cells in man^{3,4}.

The process of commitment of pluripotent stem cells to one or another line of development is poorly understood. For example, the pluripotent stem cell is not responsive to erythropoietin but at some point in development it gives rise to an erythropoietin-responsive stem cell. Such a cell is unipotent and exclusively committed to maturation along the erythroid pathway. An analogous situation holds for granulopoiesis and responsiveness to colony-stimulating activity. In a certain sense, commitment may be defined as the acquisition of responsiveness to a specific haemopoietin. Under the influence of specific haemopoietins, there is further cellular proliferation and maturation until

appropriate numbers of functional end-cells are produced. It is likely that in physiological circumstances most haematopoietic regulation occurs at the level of the committed stem cells. It has become apparent in the past few years that a diversity of cellular interactions can influence the formation of mature blood cells from stem cell precursors. The characterisation of these interactions had to await methodology for *in vitro* culture of haematopoietic cells. Of particular importance has been the development of a semi-solid gel culture system^{5,6} for the growth of monocyte stem cells and of granulocytes (CFU-G,M). (In this review the term granulocyte is taken to mean neutrophilic granulocyte, although, strictly speaking, it encompasses neutrophilic, basophilic, and eosinophilic granulocytes.) With the subsequent development of *in vitro* techniques for assaying erythroid, megakaryocyte and eosinophil precursors and B and T lymphocytes⁷, characterisation of the haematopoietic hormones which influence proliferation and differentiation has become possible. More recently, these techniques have been used to explore cellular interactions of combinations of purified cell populations or of the products of one cell lineage with the responsive cells of another lineage. Undoubtedly, some of the interactions reflect the requirements for cell growth under *in vitro* conditions; but others have physiological relevance.

Three major categories of cellular interactions have been defined thus far: the interaction between mononuclear phagocytes and a variety of other haemic cells; the interaction of T lymphocytes with progenitors of granulocytes, mononuclear phagocytes, erythroid cells, and eosinophils; and the cellular interactions of the stromal cells of haematopoietic organs.

Production of phagocytic cells

The principal phagocytic cells are the mature neutrophilic granulocytes and mononuclear phagocytes including monocytes and macrophages. The production of neutrophils is regulated by homeostatic mechanisms that normally maintain the numbers of

circulating cells within a narrowly defined range. The average neutrophil production rate for man is estimated at 1.6×10^9 cells per kg per day. Despite rapid turnover rate and high production volume, the neutrophil production system retains great flexibility in its response to inflammatory stimuli.

The production of neutrophilic granulocytes and mononuclear phagocytes are thought to be closely linked processes; both arise from a common precursor which forms colonies *in vitro* only in the presence of the haematopoietic factor, colony-stimulating activity (CSA)^{8,9}. Increasing evidence suggests that CSA is also an important controlling factor *in vivo*¹⁰. Differentiation along one of the alternative cell pathways may be controlled by the type of CSA or its concentration. Furthermore, there seems to be a family of related glycoproteins with CSA activity, some of which have specificity for different target cells¹¹.

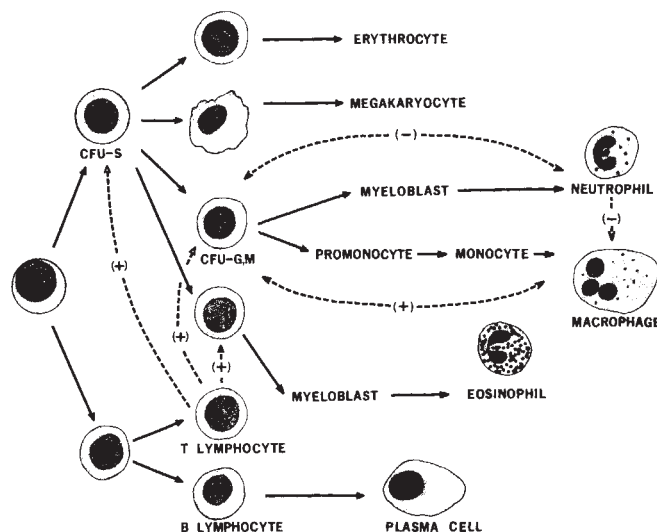


Fig. 1 Granulopoiesis is thought to be regulated by glycoprotein hormones (colony-stimulating activity, CSA) elaborated by monocytes, macrophages, and antigen-stimulated T lymphocytes. CSA acts on the committed myeloid stem cell (CFU-G,M) giving rise to both the neutrophilic granulocyte and the mononuclear phagocyte cell lines. Recent evidence also suggests that products of mature neutrophils may influence the production of both CSA and inhibitors of CFU-G,M by macrophages. If granulocyte chalone exist, they may also inhibit CFU-G,M. Stimulatory interactions are denoted by (+), possible inhibitory interactions by (-).

Only the placenta, certain fetal tissues, mononuclear phagocytes, and stimulated T cells have consistently been shown to serve as a source of CSA for human cells. Mononuclear phagocyte stimulation of granulopoiesis by CSA was the first important haematopoietic cellular interaction to be defined. The following features of this interaction are known: (1) blood monocytes, alveolar, hepatic and peritoneal macrophages all produce CSA¹²⁻¹⁴; (2) malignant monocytes of both mouse and man seem to retain CSA-producing capacity as long as some degree of monocyte differentiation is retained^{15,16}; and (3) production of CSA is modulated by exposure of cells to endotoxin, PPD, zymosan, glucan and poly I:C (refs 17, 18). The production of CSA by mononuclear phagocytes has led to a concept of positive regulation of granulopoiesis and monocytopenia by products of the responding cells themselves (Fig. 1).

Another important source of CSA is the T lymphocyte. Early observations of granulopoiesis in mice indicated that serum CSA levels were increased in primed animals exposed to antigen. It was subsequently demonstrated *in vitro* that antigen- and mitogen-stimulated T lymphocytes produce CSA, thereby providing an explanation for the *in vivo* phenomenon¹⁹⁻²¹.

Monocyte- and lymphocyte-derived CSA are similar and have higher molecular weight components than placental CSA⁹. We have recently established from a patient with a T-cell variant of hairy cell leukaemia (leukaemic reticuloendotheliosis) a line of T cells that produces potent CSA²². Production is increased, particularly after exposure of the cells to phytohaemagglutinin (Table 1). This human lymphocyte-derived CSA is sensitive to trypsin and is bound by concanavalin A (Con A).

We conclude that intense antigenic stimulation may stimulate neutrophilic granulopoiesis and monocytopenia by an interaction with T lymphocytes mediated by a particular species of CSA. Certain malignant T cells retain this stimulatory capacity.

Negative feedback

We have considered positive controls of the production of phagocytes. Various investigators have also emphasised theoretical reasons why granulopoiesis should be under negative feedback control and have adduced evidence for the existence of granulocyte chalones^{23,24}. Chalones are defined as tissue-specific, species-nonspecific products of differentiated cells that selectively inhibit early cells of the same lineage²⁵. A putative neutrophilic chalone would be a product of mature neutrophils that would specifically and reversibly inhibit the proliferation of neutrophil precursors but not other dividing cells. Although an inhibitory effect of human granulocyte chalone on human acute leukaemia has recently been reported²⁶, the existence of granulocyte chalones is controversial. For example, we were unable to find neutrophil-inhibitory products that demonstrate the requisite cellular specificity^{27,28}.

Another category of possible negative regulators of myeloid stem cells is that of neutrophil-derived factors that inhibit cells producing CSA. Broxmeyer *et al.*^{29,30} reported that crude extracts of normal, but not chronic myelocytic leukaemic, neutrophils inhibit the production of CSA by monocytes and macrophages. They therefore postulated that negative feedback from the normal mature neutrophil acted at the level of CSA generation and not at the CFU-G,M level.

Prostaglandins of the E series (PGE₁ and E₂) are potent inhibitors of CFU-G,M proliferation (activity at 10^{-10} M), whereas those of the F series are often stimulatory. The effect of PGE can be overcome with CSA. Human monocytes and mouse macrophages produce both CSA and PGE. High concentrations of macrophages inhibit CFU-G,M growth, and this inhibition is overcome with indomethacin^{31,32}. Based on these observations, Kurland and Moore have evolved an elaborate scheme whereby granulopoiesis is modulated by macrophage generation of stimulatory and inhibitory products which in turn is modulated by neutrophil inhibitors and CSA²⁹⁻³². While this scheme remains tentative, the best-defined cellular interaction in granulopoiesis is the positive stimulation of granulopoietic progenitors by products of mononuclear phagocytes and stimulated T lymphocytes (Fig. 1).

Erythropoiesis

The primary control of erythrocyte production is determined by a balance between tissue-oxygen needs and the capacity of the oxygen delivery system, and is mediated by erythropoietin. Recent studies suggest that interactions between erythroid precursors and other cells, particularly the T lymphocyte, may also modulate erythropoiesis.

Two general categories of erythroid precursors have been identified *in vitro*; first, the erythroid colony-forming unit (CFU-E), which is an immediate precursor of the differentiated normoblast and is capable of only three to six divisions; second, the burst-forming unit (BFU-E), which is a more primitive committed erythroid stem cell with potential for many more divisions. The CFU-E is thought to arise from the BFU-E³³. The CFU-E is sensitive to erythropoietin whereas the BFU-E is relatively insensitive. In the intact animal BFU-E proliferation

is influenced by stimuli to marrow regeneration but not by changes in erythropoietin concentration³⁴. BFU-E normally circulate in the peripheral blood of man and are found in the null-cell mononuclear leukocyte fraction³⁵. When T cells are removed from this fraction, burst formation is diminished; when T cells are restored, so are BFU-E. Other evidence for T-cell-erythropoietic interactions comes from studies of diffusion chambers containing proliferating haematopoietic cells implanted intraperitoneally in mice³⁶. Phytohaemagglutinin (PHA)-treated marrow cells or thymocytes added to bone marrow cell suspensions in such chambers greatly stimulate erythroid colony formation (Table 2). Pretreatment with anti- θ serum and complement eliminates the stimulatory effect of the mitogen.

A third line of evidence for a T-cell-erythroid interaction comes from a report that infusion of normal littermate bone marrow depleted of T cells does not cure the genetically determined anaemia of the W/W^v mouse, whereas addition of normal T cells to the marrow results in cure⁴⁰. A fourth line of evidence comes from the observation that medium conditioned by an established T-cell line²² has erythroid burst-promoting activity. The conditioned medium potentiates the effects of erythropoietin but cannot substitute for the hormone (Table 3). Erythroid burst-promoting activity is reported to be separable from CSA activity in other systems⁴¹.

Several types of evidence therefore suggest that T lymphocytes and their products affect early erythroid progenitors and may thereby interact with erythropoietin in controlling erythropoiesis. Less strong evidence supports a role for mononuclear phagocytes in erythropoiesis. The evidence is essentially morphological and derives from the observation of macrophage-like cells within the bone marrow in the centre of islands of developing erythroid precursors⁴². The effect, if any, of macrophages may be mediated by the prostaglandins which they produce, as PGE_1 causes increased elaboration of erythropoietin by the kidney and has direct effects on stimulating glucosamine uptake and iron incorporation into red-cell progenitors. PGE_1 also stimulates erythroid colony formation *in vitro*. It has recently been suggested⁴³ that erythropoietin is produced by macrophages in the liver, the major source of extrarenal erythropoietin^{44,45}. Alternatively, the generation of erythropoietin in the liver may depend on PGE_1 production by the Kupfer cell, which in turn regulates erythropoietin elaboration by hepatocytes. Circumstantial but not direct evidence therefore links mononuclear phagocytes to erythropoiesis.

Lymphocyte-macrophage interactions

The interactions between mononuclear phagocytes and T and B lymphocytes in antigen processing in the afferent limb of the immune response are so well documented⁴⁶ that it is only worth noting their cardinal features. First, they generally require close contact between the mononuclear phagocyte and the responding lymphoid cell. Intact viable mononuclear phagocytes are necessary for optimal response. Surface antigenic identity between the mononuclear phagocytes and the subsequently responding lymphoid cell is generally necessary. Mononuclear phagocytes potentiate the production of biologically active lymphokines, as well as B-lymphocyte antibody responses.

Table 1 Production of CSA by an established human T-cell line

Addition to culture	Duration of culture (d)	Colonies per 10^5 marrow cells
—	2	130 ± 22
—	6	105 ± 20
PHA	2	260 ± 18
PHA	6	272 ± 24

Conditioned medium was prepared from a continuously growing human T-cell line cultured in the presence and absence of PHA and assayed for its ability to stimulate granulocyte-macrophage colonies from non-adherent human bone marrow cells.

Finally, in at least some *in vitro* culture systems, the requirement for mononuclear phagocytes can be replaced by mercapto-ethanol and possibly other thiols.

The interaction between T lymphocytes and mononuclear phagocytes also operates in the reverse direction: T cells can influence mononuclear phagocyte proliferation, differentiation, and activation^{46,47}.

Eosinophilopoiesis

Basten *et al.*^{48,49} demonstrated a role for large lymphocytes in the mediation of the eosinophilic response to *Trichinella* infection in rats. Irradiated animals developed an eosinophilia in response to parasitic challenge only when reconstituted with lymphocytes as well as bone marrow cells. Marrow alone did not suffice. When lymphocytes from sensitised donors were used together with normal marrow cells, a 'secondary' type of eosinophilic response occurred. In other experiments, neonatal thymectomy significantly reduced the capacity of both mice and rats to mount an eosinophilia in response to antigen⁵⁰.

These studies in intact animals have been refined by studies in diffusion chambers implanted in mice and by *in vitro* culture systems^{51,52}. Ruscetti *et al.* added medium conditioned by sensitised splenic lymphocytes and *Trichinella spiralis* antigen to marrow cells from *Trichinella*-infected mice. They observed a three- to fourfold increase in eosinophils in liquid culture. The response was antigen specific as neither pokeweed mitogen nor PPD stimulated the release of eosinophil-stimulating factor. Collectively these experiments suggest that thymus-derived or

Table 2 Stimulation of erythroid colonies from murine bone marrow in diffusion chambers implanted in normal mice

Cells added to chambers	Erythroid colonies per chamber
Bone marrow	1.6 ± 0.8
Bone marrow + PHA	31.9 ± 7.2
Bone marrow + thymocytes	1.7 ± 1.2
Bone marrow + PHA + thymocytes	63.0 ± 22.0

Bone marrow cells (1×10^5) or thymocytes (2×10^4) were incubated for 30 min with or without PHA (0.01 ml per ml) and washed twice. In all cases 100,000 bone marrow cells were implanted in Millipore chambers containing a plasma clot. Erythroid colonies were counted 3 d after peritoneal implantation of chambers in host mice (ref. 36, and E. Niskanen, unpublished observations).

thymus-dependent cells may modulate eosinophilopoiesis in response to antigenic challenge. In addition, T-lymphocyte products may influence eosinophil migration⁵³. The granulopoietic and eosinophil responses to the soluble products of sensitised T lymphocytes are probably independent, as studies from *in vitro* culture of marrow from children with congenital neutropenia and other observations⁵⁴ support the notion of separate committed stem cells for neutrophils and eosinophils.

Cellular interactions with pluripotent stem cells

Relatively little is known about the factors influencing proliferation of pluripotent stem cells (CFU-S) or their commitment to a single line of differentiation. Under normal circumstances CFU-S do not actively proliferate. With increased demands for haematopoiesis, as after subtotal irradiation, the CFU-S compartment begins to expand. The signals for proliferation in the intact animal are not known. *In vitro*, PGE_2 has been reported to induce CFU-S to enter the proliferative cell cycle⁵⁵. PGE_1 is one of the biologically active products of the macrophage and, in theory, is a potential mediator of several haematopoietic cell interactions.

Table 3 Erythroid burst-promoting activity of medium from a human T-cell line

Addition to culture	BFU-E per 2×10^5 cells*
None	0
Epo, 3 μ l per ml	13 ± 2
Epo, 3 μ l per ml + T-cell medium, 50 μ l	42 ± 3

*Light density human peripheral blood leukocytes.

Cerny^{56,57} described another possible interaction. He reported that the number of CFU-S increased in spleen cell suspensions treated with phytohaemagglutinin (PHA). The effect was ablated by pretreatment with anti- θ serum and complement. Furthermore, conditioned medium from PHA-treated spleen cells increased the numbers of CFU-S in bone marrow two- to threefold. Pritchard⁵⁸ showed that thymocytes augment spleen colony formation when injected with marrow cells. Recent observations suggest that thymectomised mice or nude mice deficient in T cells may also demonstrate abnormalities of haematopoietic stem cell function⁵⁹. If corroborated, these many observations suggest the existence of yet another T-cell interaction with haematopoietic stem cells.

Stromal cell interaction

Another important class of haematopoietic interactions is that between proliferating haematopoietic cells and the stromal cells of blood-forming organs. At present, this class of interactions is the least understood. After transplantation into irradiated syngeneic mice, haematopoietic stem cells proliferate with a doubling time of 20–25 h. The major sites of cellular proliferation are the spleen and the medullary cavity. The rate of stem cell replication and the direction of differentiation are partly determined by factors derived from the organ of lodgment and collectively referred to as the haematopoietic inductive microenvironment (HIM) or the 'stroma'. Some of the factors seem to be under genetic control^{60,61}.

The murine bone marrow and spleen stroma can support differentiation of pluripotent haematopoietic stem cells along four pathways: erythroid, neutrophilic, eosinophilic, and megakaryocytic^{60,61}. Splenic haematopoiesis, however, is prominently erythroid, whereas granulopoiesis is more evident in the bone marrow. The effects of the stromal microenvironment on stem cell differentiation are presumed to be mediated by short-range cellular interactions.

An elegant and clear demonstration of the effect of the HIM on haematopoietic cell differentiation was provided by Wolf and Trentin⁶⁰. They implanted plugs of bone marrow stroma in the spleen and studied the pattern of growth of transplanted haematopoietic stem cells growing across the junction of such implants. On the spleen side, the colonies were predominantly erythroid, and on the marrow side, predominantly granulocytic. Subsequent investigations have suggested that an abnormal HIM for erythroid differentiation is responsible for the genetically determined anaemia of *sl/sl^d* mice^{37–39,61}.

There have been few attempts to study organ influences of haematopoiesis *in vitro*. Metcalf found that lymphocytes and thymocytes potentiated colonial bone marrow growth *in vitro*. Subsequently, Chan and Metcalf observed that femoral bone shafts could condition medium to support the growth of marrow colonies⁶². Dexter *et al.*^{63–65} have recently developed a liquid culture system in which proliferation of mouse haematopoietic stem cells can be maintained *in vitro* for long periods. In this system, marrow-derived adherent cells established in culture for three weeks are inoculated with freshly isolated bone marrow cells. The latter subsequently proliferate for several months and retain viable CFU-S and CFU-G,M. Their proliferation depends upon the adherent population comprised of phagocytic mononuclear cells, or 'epithelioid' cells, and giant fat-containing

cells which seems to provide an *in vitro* microenvironment. Within this adherent layer, extensive cellular interactions occur. Marrow-derived adherent cells from a particular mouse strain can stimulate the growth of haematopoietic cells from genetically incompatible, as well as syngeneic, mice. Dexter and Moore have recently shown functionally abnormal adherent feeder layers derived from *sl/sl^d* mice⁶⁵.

We have also used suspension and agar cultures of mouse bone marrow to study the effects of organ stromal factors on cellular proliferation and differentiation *in vitro*⁶⁶. Bone, spleen, and thymus fragments from irradiated mice were placed in contact with syngeneic bone marrow cells growing in suspension culture. Neutrophilic granulocyte numbers were greatest in the presence of bone or spleen. The stromal microenvironment of those tissues thus influences haematopoietic cell differentiation as well as proliferation *in vitro*, possibly by means of a CSA-like factor.

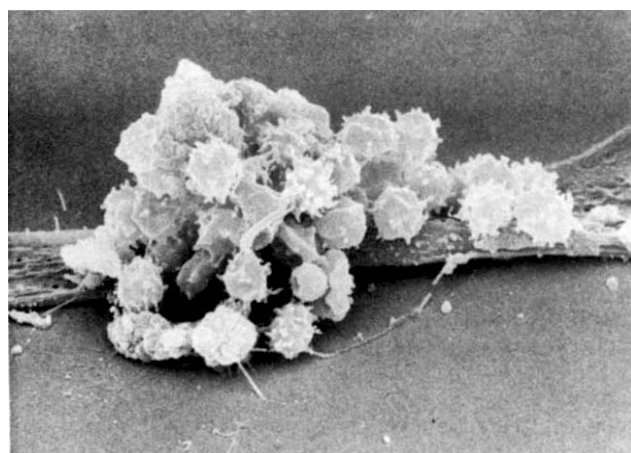


Fig. 2 Scanning electron photomicrograph of cells in long-term culture of human bone marrow. Adherent cells are established in culture before the system is 'recharged' with a fresh inoculum of bone marrow cells. The small round cells proliferate on the surface of larger adherent cells for several weeks and can be assayed in gel-matrix culture as CFU-G,M and CFU-E (photograph courtesy of Dr William Hocking and Shirley Quan of UCLA).

Recently the culture system developed by Dexter *et al.*^{63–65} has been applied to the cultivation of human bone marrow. In this system, CFU-G,M and CFU-E are preserved for several weeks. As in the murine system, large stromal cells as well as smaller round proliferating cells are in intimate contact in the growing cultures (Fig. 2). The interactions between these cells which are crucial for proliferation and differentiation are still undefined. Nevertheless, these new culture techniques do provide the means to explore stromal cell interactions.

Leukaemia

In acute myeloid leukaemia, abnormal haematopoiesis results in anaemia, thrombocytopenia, and decreased granulopoiesis. Are the normal haematopoietic cells replaced by leukaemic cells in the blood-forming organs or are they suppressed by humoral factors produced by leukaemic cells? The latter explanation is suggested by cytogenetic studies which reveal that normal haematopoietic clones are present but do not expand until the leukaemic cell mass is reduced by chemotherapy.

Leukaemic cells co-cultivated with normal marrow cells suppress granulopoiesis^{67,68}. The basis of this may be in the high molecular weight inhibitor of normal human granulocyte stem cells that we have identified in a previously described myeloid leukaemia cell line^{69,70}. This inhibitor also affects mitogen-stimulated human lymphocytes but not erythroid precursors,

normal fibroblasts, or various epithelial cell lines (Table 4). It has a molecular weight greater than 100,000, demonstrates species specificity, adsorbs to Millipore filters, and is inactivated by heat at 56 °C after 30 min. Its effect on sensitive stem cells is reversible in a time- and concentration-dependent fashion. It does not seem to affect either the S-phase of the cell cycle or mitotic events. We do not yet know if similar inhibitors are present in clinical leukaemia.

Another consideration is whether leukaemic cells retain responsiveness to normal cellular interactions and haematopoietic hormones. In general, human acute myeloid leukaemic cells in agar gel form small clusters of 4 to 20 cells rather than true colonies of 50 to several hundreds of cells. Nor is there much evidence of cytodifferentiation *in vitro*, although many leukaemias show both enhanced cluster and colony formation in response to CSA. It has been speculated that acute myeloid leukaemia sometimes results from a defect in the haematopoietic environment and its controlling hormones⁷¹; however, based on the observations cited and the demonstration that leukaemic cells retaining monocytic features can make CSA, it seems unlikely that the primary abnormality in leukaemia resides in the hormonal environment. The fundamental defect seems to be a loss of the ability to differentiate in response to normal hormonal signals and cellular interactions.

Concluding remarks

A variety of cellular interactions control the production of blood cells in haematopoietic organs. These interactions include the involvement of T lymphocytes in granulopoiesis, monocytopenia and erythropoiesis; and mononuclear phagocyte interactions in granulopoiesis, in T-lymphocyte differentiation, and perhaps in erythropoiesis. The stromal cells of haematopoietic organs provide a unique microenvironment which influences the development of stem cells.

The rich diversity of haematopoietic cellular interactions may serve as a model for interactions in other tissues and organs comprised of multiple cell types. Blood cell interactions have been elucidated *in vivo* in animal models and *in vitro* in recently developed culture systems applicable to human studies.

For several years cellular interactions affecting haematopoietic cell proliferation and differentiation have been described

in terms familiar to cell biologists: for example, the activation of macrophages by soluble products of lymphoid cells is well established⁴⁶. More recently, permanent lines of haematopoietic cells have provided tools for studying induced differentiation of eukaryotic cells at the molecular level^{72,73}. It is likely that these model systems will provide the ultimate explanations of the influence of one cell upon the proliferation and differentiation of its neighbour. It is also possible that the techniques used to examine these interactions may have unanticipated benefits for the treatment of human disease. For example, it has recently been reported that mixtures of haematopoietic stem cells cultured for long periods *in vitro* can change their antigenic

Table 4 Inhibition of proliferating cells by medium from a myeloid leukaemia cell line

Target cells	Inhibitor concentration v/v	Inhibition of growth (%)
Human CFU-G,M	1:100	98 ± 2
Human CFU-E	1:10	64 ± 4
	1:100	6 ± 4
Mouse CFU-G,M	1:10	74 ± 6
	1:100	23 ± 5
Human fibroblasts	1:10	2 ± 2
Human epithelioid cells 253J	1:10	0 ± 2
Human T lymphocytes (PHA)	1:10	82 ± 6

characteristics and subsequently proliferate in host animals which would ordinarily reject their growth⁷⁴. This observation may have important implications for the biology and technology of human bone marrow transplantation for aplastic anaemia and malignant disease. Currently such transplantation is restricted to donors and recipients possessing haematopoietic cells which are closely similar in surface antigenic structure. The possibility that one could artificially alter that structure is challenging.

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articles

Superluminally expanding radio sources and the radio-quiet QSOs

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We propose a model of apparent superluminal expansion based on straightforward relativistic motion. The model gives a natural explanation of the ratio of radio-quiet to radio-loud quasars.

THE expansion of the double structures of quasars revealed by VLBI^{1,2} at apparent separation speeds exceeding $2c$, has posed an intriguing problem; the numerous suggestions that have been made for its solution have been classified and discussed by Blandford *et al.*³. Theories of the type they call 'ballistic', in which radio-emitting matter is projected at $v \sim c$ in directions making small angles θ with the direction towards the observer, have been current for a long time, and a version involving spherically symmetrical relativistic expansion was originally devised by Rees⁴ to account for the rapid variability of certain powerful radio sources. In a two-component version of the theory, the approaching component has apparent transverse speed $v \sin \theta / (1 - (v/c) \cos \theta)$ and apparent power $P_0 \gamma (1 - (v/c) \cos \theta)^{-(3+\alpha)}$; for the receding component v is replaced by $-v$ (ref. 5) (P_0 is the radio power per Hz in the component's rest frame, and α its spectral index in the sense $P \propto \nu^{-\alpha}$). The maximum apparent transverse speed occurs when $\sin \theta = 1/\gamma$ and is γv ; thus an apparent speed of $5c$ calls for γ a little > 5 .

The traditional difficulties with this type of model are: (1) if their intrinsic powers are at all similar, the receding component has a flux density very much smaller than that of the approaching component; even for modest v/c this ratio is large (see ref. 6) and for $\theta \sim 1/\gamma$ with $\gamma \geq 2$ the receding component should be quite unobservable. This totally disagrees with the reported fairly symmetrical double structure. (2) In a randomly orientated sample of sources, very few have axes sufficiently close to the line of sight to reach large v_{apparent}/c ; for example, for $\gamma = 5$ the proportion with $\sin \theta < 1/\gamma$ is 2%. However, superluminal velocities are observed in a large proportion of the quasars that have been observed systematically by VLBI.

Lynden-Bell⁷ has shown one way out: he postulates that the ejecta merely excite the surrounding ($\sim$ stationary) matter to emit radio waves, thus avoiding difficulty (1), and removes (2) by using a large value of Hubble's constant, so that only modest γ 's are needed.

Another way is to suppose that the observed VLBI 'doubles' consist of approaching components only. In this scheme the appearance of an expanding double is due to a bright knot in the approaching stream, together with radio emission continually

regenerated near the quasar nucleus; a relatively small total energy is enough to provide observable radio emission from a very compact source near the nucleus, but the amount of radio emission there is limited by synchrotron self-absorption, so that we may expect the radio emission close to the nucleus to fluctuate rather less than the energy supply. Difficulty (1) is then irrelevant, and (2) is attributed to a selection effect, sources approaching with high γ and small θ being observed often because of their high apparent radio power. The much larger number of similar sources with large θ are then to be identified with some or all of the radio-quiet quasars, which are optically very similar to quasi-stellar radio sources (QSRS). We have favoured this model for some time, but the stimulus to working out its consequences more seriously came from recent work at Caltech⁸⁻¹² which shows that, in six out of the eight cases observed, the VLBI structures in fact consist of a (so far) unresolved, self-absorbed component at one end, and a single 'jet' (with a steeper spectrum) emerging from it.

The six sources showing clear core-jet structure are 3C120, 3C147, 3C273, 3C286, 3C345, 3C454.3; the two other sources, CTA102 and 3C380, show no striking asymmetry, but have so far been observed at only one frequency.

Here we define the nature of the model we propose; compute the ratio of total optical QSO numbers to QSRs that it requires; examine the problem of compact central sources associated with large classical doubles; and point out some other observational consequences of the model.

The model

We postulate that the radio-emitting objects are ejected often, or continually, but by no means steadily, along one axis. Thus there is always some radio emission close to the QSO, but all the radio-emitters on the approaching side are moving with similar velocities. (A stationary nuclear component would have flux density independent of orientation, and thus defeat the model through difficulty (2)). We must suppose, however, that much of the light comes from a stationary source, as the line emission does not have large v/c (otherwise blueshifts would occur), and the line: continuum ratio does not vary really drastically from one quasar to another. The model would not be inconsistent with an appreciable fraction of the continuum originating in the relativistic jet in some cases, and indeed such an interpretation would be required if a strong trend for greater optical variability among QSRs than among QSOs as a whole (see ref. 13) is confirmed.

We need a range of γ extending up to at least 7 (to account for v_{apparent} in 3C345), and down to 1.5 or less to account for the less powerful but relatively common central components of classical double radio sources. Though most of our calculations refer to quasars with only a single value of γ (and also of P_0 , α , and optical luminosity) and we concentrate on the distribution in θ , a more thorough treatment will require model-building with distributions of these parameters.

Blandford has pointed out that, if a succession of pairs of components is ejected, and each lasts a given proper time (after which its radio emission fades), then at any instant we observe more of those on the receding side than on the approaching side; thus one Doppler shift factor disappears from Ryle and Longair's formula⁵ for the apparent radio power, that is, the exponent becomes $(2+\alpha)$ instead of $(3+\alpha)$. The VLBI observations indicate that we are generally observing several components at once, so that our model will generally correspond more closely to Blandford's picture than to that of Ryle and Longair; hence we adopt an exponent $(2+\alpha)$ in our formulae, and show numerical results corresponding to the strict two-component model by giving α values 1.0 in excess of normal spectral indices.

The number of radio-quiet QSOs

In our model, the approaching half of the radio source gives essentially all its observed flux density S ; if it lay transversely in the plane of the sky, it would have given a flux density S_t , where

$$S_t = S(1 - (v/c) \cos \theta)^{2+\alpha} \quad (1)$$

(Thus a symmetrical double source with $\theta = 90^\circ$ would have flux density $2S_t$; as in our model the radio emission is very faint for sources with large θ , we shall ignore this fine distinction between S_t and $2S_t$.) We introduce S_t because it depends on distance like S , but is independent of θ , and for our standard quasar we can therefore regard S_t as a measure of the optical flux density.

For our rough estimate, we take the optical differential source count for quasars to be a power law

$$dN = K S_t^{-\beta-1} dS_t \quad (2)$$

The density of quasars in the S, S_t plane is then given by

$$dN = K S_t^{-\beta-1} dS_t d(\cos \theta) \\ = \frac{Kc}{v(3+\alpha)} S_t^{-\beta-1/(2+\alpha)} S^{-1/(2+\alpha)} dS_t dS \quad (3)$$

(using equation (1)) in the band between the lines $S_t = S$ and

$$S_t = S(1 - v/c)^{2+\alpha} = fS \quad (4)$$

Note that $f \ll 1$ even for modest values of γ : $f \leq 0.01$ for $\gamma = 2$ and $f \approx (2\gamma^2)^{-(2+\alpha)}$ for large γ . If we set optical and radio limits of detection at S_0 and S_r , respectively, such that an appreciable fraction of the sources with $S > S_r$ are optically identified (Fig. 1), then $S_r \gg S_0$ and we find:

Fraction of QSRs with $S > S_r$ that are not optically identified =

$$(1 - f^{\beta-1/(2+\alpha)})^{-1} \{1 - \beta(2+\alpha)(fS_r/S_0)^{\beta-1/(2+\alpha)} \\ + ((2+\alpha)\beta - 1)(fS_r/S_0)^\beta\} \quad (5)$$

Fraction of QSO with $S_t > S_0$ which are also QSRs with $S > S_r$ =

$$\frac{c}{v} \left\{ \frac{\beta(2+\alpha)}{\beta(2+\alpha)-1} (S_0/S_r)^{1/(2+\alpha)} - (1 - v/c) - \frac{1}{\beta(2+\alpha)-1} (S_0/S_r)^\beta \right\} \\ \approx \frac{1}{2\gamma^2} \left\{ \frac{\beta(2+\alpha)}{\beta(2+\alpha)-1} (S_0/fS_r)^{1/(2+\alpha)} - 1 \right\} \quad (6)$$

We can safely set the first factor in equation (5) to unity; eliminating the quantity (fS_r/S_0) between equations (5) and (6) then lets us present the relationship between the fraction of

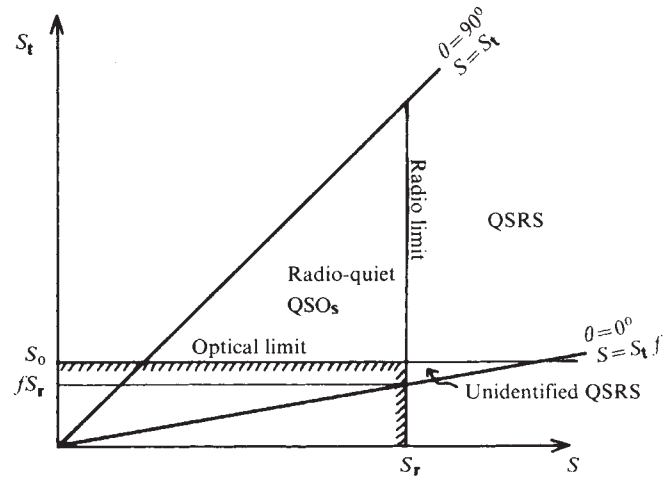


Fig. 1 The S (radio flux density) – S_t (essentially optical flux density) plane. Sources exist between the lines marked $\theta = 0^\circ$ and $\theta = 90^\circ$.

unidentified QSRs and the fraction of QSRs in an optically selected sample in the simple form shown in Fig. 2.

Evidently, if most of the QSRs are optically identified (which is certainly true of flat-spectrum quasars), the ratio of total QSOs to QSRs is several $\gamma^2:1$; if all are identified ($S_0 < fS_r$) the ratio exceeds $2\gamma^2\{\beta(2+\alpha)-1\}$ that is it is $>4\gamma^2$ for $\beta = 1.5$, $\alpha = 0$. The numerical factor does not depend critically on the proportion of unidentified quasars, or on β or α ; thus if γ is typically 5, we expect about 1% of an optically selected sample to be radio sources. Furthermore, we expect the proportion to rise very slowly if we look with more sensitive radio telescopes; equation (6) shows that the proportion of QSRs rises roughly like $S_r^{-1/(2+\alpha)}$. This feature, too, is qualitatively entirely in agreement with observational experience.

The distribution of apparent velocities

We can gain insight into the apparent velocity distribution in a radio-selected sample by working in the approximation $\gamma \gg 1$, $\theta \ll 1$. To this approximation, the proportion of sources with radio flux density $S > S_r$ having $v_{\text{apparent}}/c > \gamma \sin \chi$ is

$$(\cos \frac{1}{2}\chi)^{2\beta(2+\alpha)-2} - (\sin \frac{1}{2}\chi)^{2\beta(2+\alpha)-2} \quad (7)$$

With $\beta = 1.5$, $\alpha = 0$ ($\beta = 1.8$, $\alpha = 1.5$), 87% (69%) of sources have $v_{\text{apparent}}/c > 0.5\gamma$ and 60% (31%) have $v_{\text{apparent}}/c > 0.8\gamma$. Thus apparent velocities comparable with γc are indeed expected to be very common in a radio-selected sample. (The exact figures are 82% (62%) and 39% (16%) at $\gamma = 2$, so the approximation is a fair guide even for small γ .)

Central components of classical double sources

In classical double radio sources, where the central component generally contributes little to the total flux density (especially at low frequencies), we should have a sample with random orientation of axes. However, a substantial fraction of such sources have central components whose properties seem to be qualitatively similar to compact sources without extended structure, and which are roughly aligned with the large-scale double in the few cases where VLBI measurements exist. No direct measurements of apparent expansion velocities exist yet. (We must exclude sources with extended structure on one side only, such as 3C273, also 3C345 and 3C454.3 (ref. 14) as in these, the extended structure might have substantial v/c). However, a few central components are known to be radio variables.

Clearly, very few of these central components can be sources with $\gamma \geq 5$, as we have seen that for each such source there must be several γ^2 'radio-quiet sources'—here, doubles without observable central components—whereas it is observed that

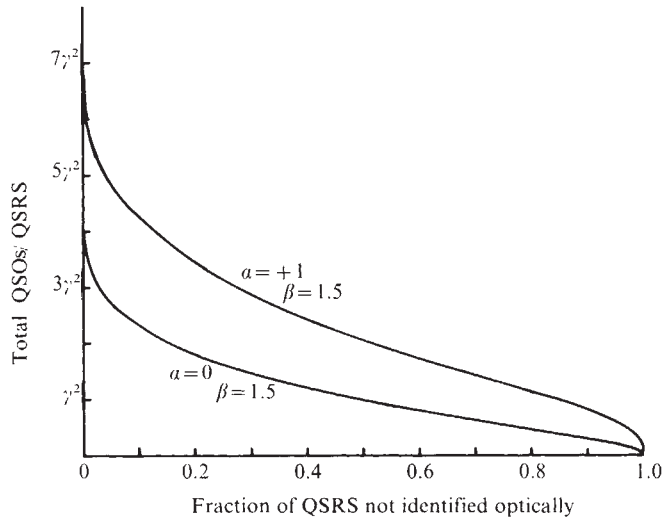


Fig. 2 The ratio of total quasars (radio quiet and QSRS) to QSRS alone, above given radio and optical limits, as a function of the fraction of unidentified QSRS in the radio-limited sample. The differential optical source count is assumed to be a power law with index $-(\beta + 1)$; the line $\alpha = 0$ represents flat-spectrum sources in mean, while the $\alpha = +1$ line represents either steep-spectrum sources or a strict 2-component model for flat-spectrum sources (see text).

~30% of 3C radio doubles (and more than half of the 3C quasars with extended double structure) have central components. We now make this argument a little more quantitative. For sources of identical γ , S_c and α , the distribution of apparent intensities due to their random orientations is given by

$$dN \propto d(\cos \theta) = \text{constant} \times S^{-1/(2+\alpha)} dS, S_c < S < S_c/f \quad (8)$$

and zero outside these limits. To estimate the largest γ we can allow as typical for central components, we compare equation (8) with the narrowest distribution we can find for S relative to some orientation-independent quantity, which is that of the ratio R of S to the flux density of the extended double structure. We use the complete sample of 166 3C sources selected at 178 MHz (listed in ref. 15). From it, we select those sources that: first, have large-scale structure on both sides of the optical position; second, are well enough resolved to yield an estimate or a useful upper limit for the flux density of the central component at 5 GHz (in some cases of doubtful interpretation, 15 GHz maps with 0.67" resolution have also been inspected); third, are identified with quasars.

We use only quasars because central components are commoner in them than in sources as a whole, so that they lead to a severer restriction on γ . (We should also exclude sources whose 178 MHz flux in extended structure alone would have fallen below the $S_c = 10$ Jy limit of the sample, but found no likely cases that had not already been excluded on other grounds.) The result (see Fig. 3) shows that a range of 64:1 in the ratio R is compatible with the observations; this corresponds to $\gamma = 2$. Even $\gamma = 2.5$ would be hard to reconcile with the observations: the total number of sources would then have to be six times the number in the top three octaves, that is, 36 instead of the 22 on our list. Of course, the true distribution is likely to contain a range of γ , extending from values close to 1 to $\gamma = 2.5$ and beyond; such a range of γ , together with a scatter in other intrinsic properties, would give a more bell-shaped distribution than equation (8).

We emphasise that there is no need to suppose that the central components of classical doubles are drawn from a population with a quite different γ distribution from that of the compact quasars; information available would allow us to ascribe the apparent difference solely to selection effects, for the central

components by themselves would make very little difference to the counts of radio sources, or of quasars.

A more serious matter is the variability of central components. The observational evidence is still very sparse, and the most striking case we know is that of 3C111 at $z = 0.049$, whose central component has been known to vary by 2 Jy in 0.4 yr (R. G. Hine, personal communication). This rate of variation would be compatible with conventional electron synchrotron emission for a component speed corresponding to $\gamma \sim 2$, or rather less if the component is itself expanding with mildly relativistic speed.

The radio source counts at high frequencies

It is well known that the radio source counts at high frequencies (2.7 and 5 GHz) deviate much less from a power law with $\beta = 1.5$ than do the source counts at 408 MHz and lower frequencies (see ref. 16). There are two ways in which this fact could be related to the present model.

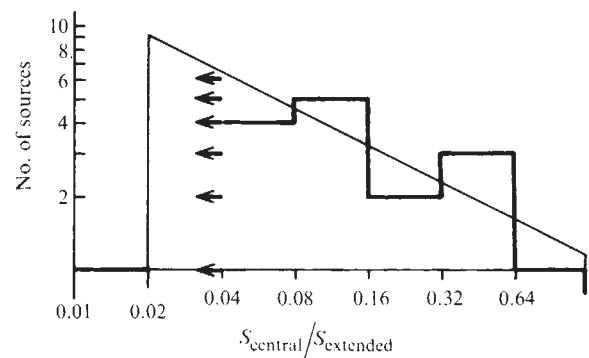
First, the distribution of S with orientation (equation (8)) introduces a broad scatter in apparent radio luminosity in addition to the intrinsic radio luminosity function. This might be expected to flatten out the hump in the graph of normalised source counts versus S (Fig. 1 of ref. 16) at high frequencies, where the source counts are dominated by flat-spectrum quasars, but not at low frequencies, where the source counts are dominated by classical doubles and orientation effects are therefore unimportant. In fact, this effect is far too small to account for the observed difference between low and high frequency counts, because the distribution in equation (8) is so much less steep than the source counts. Numerical convolution of the 408-MHz source counts (from ref. 16) with distributions given by equation (8) flattens the graph of normalised source counts slightly though noticeably at low flux densities, and changes its slope very little indeed at high flux densities.

Second, it is also well known that low-powered radio sources evolve far less strongly than high-powered radio sources. In the present model, the flat-spectrum quasars are sources of low intrinsic radio luminosity, promoted to high flux density by high speed and favourable orientation. Thus the present model may provide a link between the relatively slow evolution of flat-spectrum quasars and the relatively slow evolution of weak radio sources.

The curvature of the VLBI structure

Readhead *et al.*¹⁰ have considered the VLBI structure of radio sources which have extended components on either side of the optical object, and of sources in which the extended structure, if any, is one-sided and a dominant compact ($<0.1''$) radio

Fig. 3 Histogram of the numbers of classical double quasars with various ratios of central component flux density to extended component flux density (note log scales). The thin line is the predicted distribution for the model sources with flat spectra ($\alpha = 0$) expanding at $\gamma = 2$, fitted to the histogram; larger γ would require a larger range along the abscissa, and hence a greater number of sources without observable central components.



component is coincident with the optical object. They call these type S and type C objects, respectively, and find that, in those type S objects that have a sufficiently bright central radio component to be studied by VLBI, this structure is well aligned with the outer components (five cases). Conversely, in type C objects the compact component is generally curved (four cases). If our model is correct, the fact that curvature is seen at all implies that the opening angle of the jet is considerably less than $1/\gamma$. The curvature observed, projected on to the plane of the sky, will be the intrinsic curvature of the jet magnified by a factor $\text{cosec } \theta$, that is a factor $\geq \gamma$ according to our model. Thus our model leads us to expect much greater curvature in type C sources (large γ , small θ) than in type S sources (small γ , random θ). The curvature observed in type C sources ranges from 20° to 45° .

Conclusions

We believe that the model suggested here is consistent with the known facts about quasars. The model lacks ingenuity, and we regard that as one of its merits. The model is a fragile one; the clearest and cleanest evidence against it would be the detection of large superluminal apparent expansion velocities in the central components of even a few quasars with extended double structure. As a preliminary to looking for such evidence we hope to encourage more extensive monitoring of flux density variations in such central components.

We note that the high speeds of approach in the present model increase the limit on apparent surface brightness imposed by the onset of inverse Compton scattering; thus it is rather easier to account for rapid variability below 1 GHz^{17,18} than it would be in models in which the radio emitters are static.

Much more detailed model-building will be required to find out whether the model is consistent with all aspects of quasar statistics, such as their distribution in redshift and in optical

luminosity. We have also treated the radio components as being ejected into an infinitesimal cone at a single value of θ ; ejection into a finite range of θ could have substantial effects on the statistics. The different typical γ s required for compact quasars and quasars with extended double structure seem at first to be a very contrived feature of the model, but it seems to us that this difference can arise largely or entirely as a result of the different methods of selection. We are much less happy about the need to attribute relativistic speeds to the single-ended extended structures in 3C273, 3C345, and 3C454.3, though we are consoled by the facts that (1) these extended structures are in all three cases on the same side of the most compact components as is the jet, and (2) two such structures (in 3C273 and in 3C274 (M87)) are also distinguished from other extended radio structures by their strong optical continuum emission.

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On the origin of the universal dielectric response in condensed matter

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The observed dielectric response of most materials is seldom in accord with Debye behaviour, but has been found experimentally to exhibit a remarkable 'universality.' It is proposed that this universality is a consequence of some ubiquitous correlated states which exhibit an infrared (IR) divergent-like response to induced transitions of the polarising species.

IN a recent detailed survey^{1–4} of the dielectric response of a wide range of solids, it was concluded that the time or frequency response departs strongly from the Debye response and that it falls into a remarkably common or 'universal' pattern in which the frequency dependence of the dielectric loss follows the empirical law

$$\chi''(\omega) \propto \omega^{-n-1} \quad \text{with} \quad 0 < n < 1 \quad (1)$$

extending over several decades of frequency from the lowest audio and sub-audio range to $\omega/2\pi \approx 10^9$ Hz, in a remarkably

wide range of different materials. The corresponding time-domain response of the depolarisation current is given by the Fourier transform of equation (1) and has been known for many years as the Curie-von Schweidler law:

$$i(t) \propto t^{-n} \quad (2)$$

The universal response applies to permanent dipoles and to hopping charge carriers of electronic, polaronic and ionic nature. It is valid in solids with covalent, ionic and molecular bonds, in single crystals, polycrystalline and amorphous structures, and hence is apparently independent of the particulars of the material. At higher frequencies, in excess of 10^9 Hz, quantum effects involving lattice mode excitations and/or electronic excitations become prominent but these will not interest us in the present context.

The various types of dielectric response are summarised⁴ in Fig. 1. We note the virtual absence of the pure Debye behaviour. In dipolar materials the universal response, equation (1), is followed at low frequencies by a loss peak of either the α or the β type, while in systems dominated by hopping charge carriers

another universal response with very small values of n , typically between 0.1 and 0.3, follows at low frequencies⁵.

The totality of these experimentally ascertainable facts is not compatible with any of the numerous theories that have been proposed in the last half-century to explain the behaviour of this or that particular class of materials and the need to invoke many-body interactions to account for the generality of equation (1) has been suggested²⁻⁴. The possible connection of this behaviour with infrared divergent response has also been conjectured (K. L. Ngai, unpublished).

This state of affairs has motivated us to seek a more fundamental understanding of the universal behaviour in terms of physically simple and 'elementary' properties. We will present here several elementary principles which when combined will enable a derivation of the universal law shown in equation (1) to be made regardless of the physical, chemical and geometrical properties of the solids, and also regardless of the nature of the electrically active species responsible for polarisation, whether dipoles, electrons or ions. We will also discuss several examples of low energy excitations expected in various systems that satisfy these elementary principles. We shall derive the universal response (1) and briefly mention the possible presence of a loss peak at lower frequencies.

IR divergence and the universal law

IR divergence phenomena, although not commonly observed in physics, have been seen in several instances, notably in quantum electrodynamics as bremsstrahlung⁶, and in solid state physics^{7,8} as the peculiar shape of X-ray absorption edges of metals⁹. These examples are not exhaustive but the subjects they cover demonstrate that IR divergence is not uncommon.

The features common to systems exhibiting the IR divergence are: (1) the sudden application of a potential, or a sudden change of the potential or the hamiltonian; and (2) the availability of low-energy excitations of the system and its response to the sudden potential change dominated by the emissions of these low-energy excitations.

In the time domain, the phenomenon is the transient response¹⁰⁻¹² of the system to that abrupt change of potential. IR divergence occurs whenever the suddenly switched on potential V excites some low energy excitations, with a density of states $N(E)$ for excitation energy E , which is such that $V^2(E)N(E) \propto E$. In this instance there is an increasingly high probability of exciting decreasingly small energy excitations and this causes a power-law divergence of the response in the frequency domain, equation (1), or its time-domain equivalent, equation (2). In the X-ray edge problem in metals an absorbed X-ray photon suddenly switches on a hole-core potential V for the conduction electrons. The low-energy excitations here are the electron-hole pairs.

We shall propose later that within a broad classification of dielectrics some ubiquitous states exist that, for convenience, we shall refer to as correlated states. Low energy excitation of the correlated states with excitation energy E consists of removing an 'occupied' state to an 'unoccupied' state and is the analogue of the electron-hole pair excitation in the X-ray edge problem. Due to induced transitions of the charged particles or dipoles responsible for the polarisation of the dielectric, a potential V is turned on acting on these correlated states. Hence the response of the dielectric involves emission of correlated state excitations. We shall argue in a specific example given that the low energy excitations of these correlated states have a density of states $N(E) \propto E$, and the potential change V has little or no E dependence. Furthermore, V may be taken as suddenly applied as the polarising species of the dielectrics should undergo quantum transitions, including changes in their positions/orientations, between preferred states in an abrupt manner by hopping or jumping movements¹⁻⁴ such that the time $1/\nu$ taken by the actual transition is negligible in comparison with both (1) the time spent on average in the respective preferred states, and (2) the time characteristic of the low energy excitation of the correlated states. Condition (1) is invariably satisfied in solid

dielectrics. That condition (2) is also satisfied will become clearer after we have considered the nature of the correlated states. It then follows that the requirements for an IR divergent dielectric response of the correlated states are satisfied. The mean number p of correlated state excitations is then $p = b \int_0^{E_c} V^2 N(E) dE / E^2$, which diverges logarithmically, where E_c is the upper 'cut-off' at the correlated state excitation energy.

It is interesting that the IR divergence problem when considered in the time domain as a transient response problem¹⁰⁻¹² does lead to the time decay of the response function for large times as t^{-n} . The derivation of the complete dipolar loss peak response will be discussed elsewhere, meanwhile we discuss the correlated states in a broad classification of dielectrics.

Correlated states

We have connected the universal law, equation (1), to an IR divergent response of correlated states. For this interpretation to follow, such states must be prevalent in dielectrics and have characteristic response times long in comparison to the switching of the hamiltonian. Below, we discuss several examples of such correlated states which can reasonably be expected to be present in many dielectrics. Note, however, that although these examples are quite general, they are by no means exhaustive. Furthermore, the first class of correlated states which we discuss (those related to spin density fluctuations) is not the most general as the majority of dielectrics are diamagnetic. We have chosen this class for initial consideration because the resultant analysis facilitates the treatment of the more important classes of correlated states considered subsequently.

(1) Dielectrics with localised spins: new concepts and ideas on low-energy excitations in spin glasses (and real glasses) have been recently introduced by Anderson *et al.*¹³, Phillips¹⁴ and by Anderson¹⁵. They propose the existence of a statistical distribution of localised tunnelling levels and/or modes. In a spin glass, spins are considered as classical dynamical quantities with a potential energy surface that is a function of the simultaneously specified orientations of all the spins; local minima in the energy correspond to metastable states of the spin glass associated with different spin configurations. A tunnelling mode (TM) in a spin glass^{13,15} is defined in spin configurational space as two local minima of energy difference ΔE separated by a quantum-mechanical energy barrier. Tunnelling between one local minimum and another, if it occurs, involves the rearrangement of several spins. The 'low' temperature linear specific heat observed in spin glasses comes from TMs with energy barriers that are sufficiently great so that resonant tunnelling of spins (atoms) between the local minima does not occur, but small enough so that tunnelling between the two levels can take place during the time span of the specific heat measurement.

Let us examine the transient response of these spin TMs to a sudden potential change, V , caused by fast quantum transitions of some charged species. Tunnelling modes whose alternate states are separated by more than $\sim \hbar\omega_0$, with $\omega_0 = 10$ GHz, can be eliminated at the outset for consideration of IR divergent response as our interest is in the low frequency dielectric behaviour where ω is smaller or much smaller than 10 GHz. Also, we need to consider only the very low energy TMs with quantum-mechanical energy barriers large enough so that the characteristic response time of the TM to V is long in comparison with the jump time of the polarisable species. This class of TMs should exist. Anderson¹⁵ pointed out that as the configurations of the spins (atoms) is random, there must be very many locations (of order N ; the number of spins or sets of spins), where there are two possible configurations of very similar energies E_1 and E_2 . If E_1 and E_2 are independent random variables, then the probability $P(\Delta E)$ of finding $\Delta E = |E_2 - E_1|$ is finite as $\Delta E \rightarrow 0$. But physically this is not true because it is possible to tunnel between the two alternate levels with a tunnelling matrix element T_{12} even though this is small for the TMs under consideration. The energy level separation will be at least $\Delta E \geq |T_{12}|$; the off-diagonal matrix element between the alternate levels. For the low frequency dielectric response (that

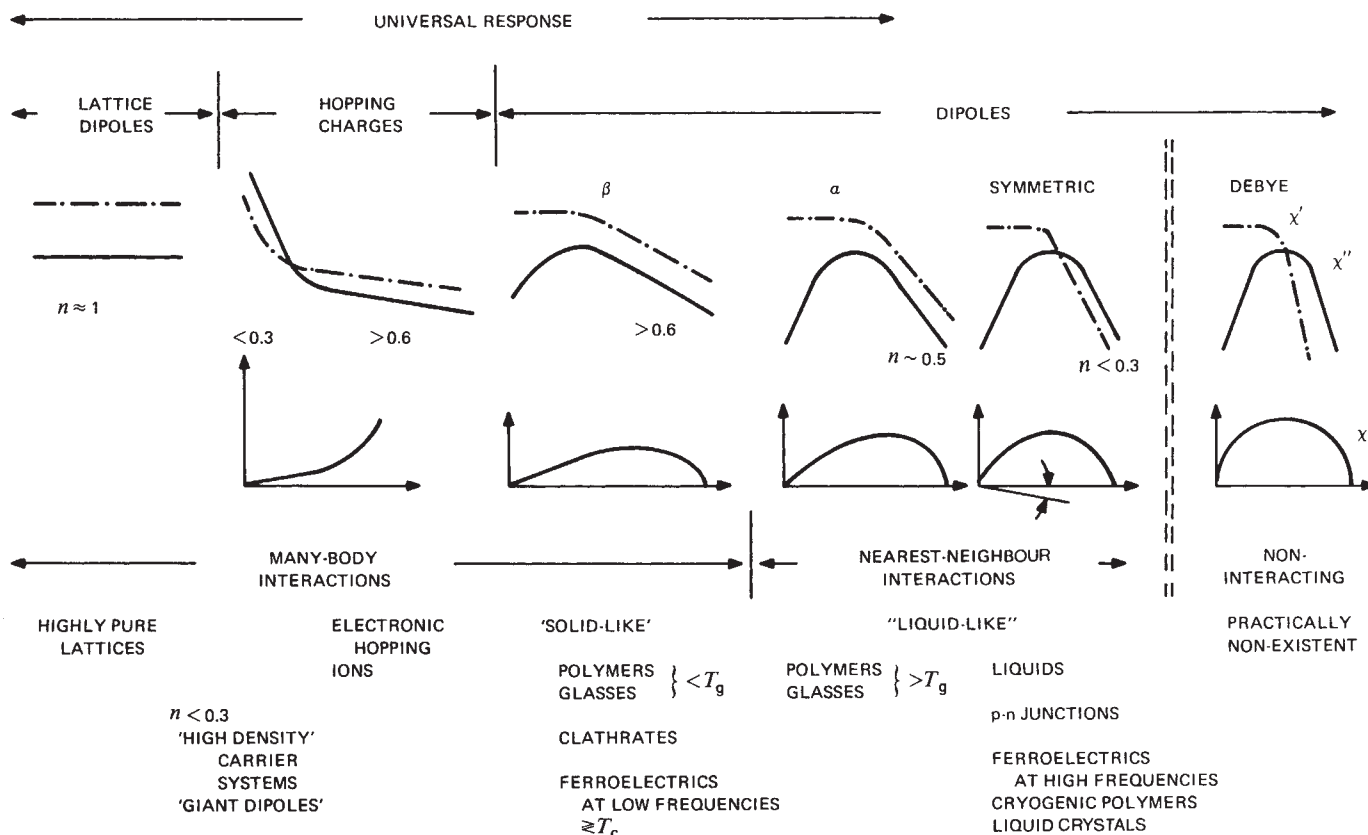


Fig. 1 A representation of the various observed types of dielectric response in the entire range of solids. The upper set of diagrams represent the shapes of the logarithmic plots of $\chi'(\omega)$ (chain-dotted lines) and $\chi''(\omega)$ (solid lines), ranging from the ideal Debye through the α and β peaks and on to the universal dependence for charged carrier systems. The limiting forms of behaviour are represented by the strong low-frequency dispersion with small values of n and by the limiting case of frequency-independent 'lattice response' with $n \approx 1$. The lower set of diagrams represents the corresponding complex χ plots. The various types of materials obeying the respective types are shown and the presumed polarisation mechanisms are indicated. For further details and source references see ref. 4.

is, < 10 GHz) we are particularly interested in the $\Delta E = |T_{12}| \rightarrow 0$ limit. It has been argued by Anderson¹⁵, that T_{12} being a complex matrix element acts like the x and y components of the random field, as it has real and imaginary parts. The probability that the mode energy ΔE lies in the interval $|T|$ and $|T| + d|T|$ is then proportional to $|T|d|T|$. Thus, the density of states of very low energy, tunnelling modes, $N(\Delta E)$, is proportional to ΔE . Now the sudden potential change that induces transitions between the two alternate levels should on the whole not depend on ΔE . Thus, within this class of tunnelling modes, the condition for IR divergence $|V_{12}|^2 N(\Delta E) = n \Delta E$ is satisfied and the universal law follows for spin-glasses. Note that here, and henceforth, (unless otherwise stated) the two states of the TMs are considered thermodynamically accessible to each other. The possible role of thermodynamically inaccessible correlated states in contributing to the divergent dielectric response will be pointed out later. Also note that quite generally systems describable, for example, by a random Hubbard model would be expected to exhibit magnetic properties describable in terms of spin glass states with an example being perhaps impurity bands in crystalline semiconductors.

(2) Dielectrics with atom-atom or molecule-molecule or ion-ion or dipole-dipole interactions: spin-spin interaction models can often be transcribed to other physical models with non-spin interactions¹⁷. Well known examples include the Ising model equivalence to a lattice gas and to a binary alloy. A lattice gas is a collection of atoms (molecules) whose positions can take on only discrete values which form a lattice. Each lattice site can be occupied by at most one atom. In general the potential energy of the system of atoms corresponds to a gas in which the atoms are located only on lattice sites and interact through a two-body potential $v(|\mathbf{r}_i - \mathbf{r}_j|)$. The correspondence between the lattice gas and the Ising model is then practically immediate¹⁸.

Consider dielectrics where atom-atom, molecule-molecule or ion-ion interactions are important. In the 'lattice' gas and/or binary alloy modelling of dielectrics with random interactions, the essential equivalence to the spin glass Ising model implies that a dielectric state corresponding to the spin glass state exists. Such dielectrics will have, in analogy to spin glasses, very low energy tunnelling modes which correspond to the change of the atomic (molecular or ionic) occupancy of several sites in getting from one energy minimum to the other. This class of tunnelling modes again should satisfy the criterion for IR divergence and hence yields the universal law. The lattice gas and binary alloy model should be good representations of many dielectrics including the class of solid state ionic conductors^{19,20} or solid electrolytes such as AgI, CaF and Na β -alumina. In fact, ionic conductivity for these solids has been calculated in the lattice gas model²¹. In the case of Na β -alumina, there is the repulsive interaction among the diffusing sodium ions and also the attractive interactions between the ions and their randomly distributed, compensating defects. These properties imply a lattice gas with random interactions. There is ample experimental evidence^{22,23} for the existence of tunnelling modes in alkali β -alumina as well as Ag β -alumina. In particular there is an excess low temperature specific heat^{23,24} contribution just as in the case of the spin glasses.

(3) Dielectrics with electron self-trapping states: the concept of local electron self-trapping largely arises from the observation that by and large if a particular electronic state is singly occupied the atom or atoms principally associated with this state will adjust their positions in such a way as to lower the energy level of this state relative to its value when unoccupied. A traditional example is provided by those ionic solids where strong local coulomb interactions between the electron and lattice constituents induces a local lattice distortion leading possibly to

the self-trapping of the electron and the formation of a small polaron. This tendency is not simply restricted to ionic materials but is much more general. Well known examples lying outside the usual small polaron mechanism are provided by the reconstruction of semiconductor surfaces^{25,26} and electron pairing states in amorphous glasses^{27,28}. The origin of the self-trapping in these instances is essentially a dehybridisation energy^{25,26,29,30} arising from concepts inherent in covalency and stereochemistry. The electron-lattice interaction may be strong enough to make it energetically much more favourable to self-trap electrons in pairs rather than singly and such systems have been described by Anderson²⁷ through a negative U Hubbard model. Let us restrict our attention for the moment to the negative U electron pairing states which should be the physically most important due to the prevalence of diamagnetic systems in nature. In this instance several subgroups of possible correlated states can be identified with properties leading to the desired behaviour in " where, in each case, the response time of these states is expected to be acceptably slow due to the lattice coordinates involved.

Consider first those systems where there are a continuous density of pairing states, the number of which exceeds the number of available electrons. In these instances one can envision very low energy TMs corresponding to different arrangements of the electrons over the pairing centres. By direct analogy with the spin glass TMs these electron-pair TMs should exhibit the desired properties; that is $N(\Delta E) \propto \Delta E$, $V(E) = \text{constant}$.

Another not completely orthogonal subgroup of bipolaron derived TMs that could contribute to the ω^{n-1} behaviour of χ'' is realised when the system is such that bipolaron states are essentially degenerate with, for example, non-distortive extended states. Furthermore, in those instances where the coulomb repulsion dominates, heavily favouring single self-trapping of the electron, various subgroups of correlated states can be identified which are completely analogous to the bipolaron ones outlined above. Intermediate subgroups can also be defined where, for example, one envisages very low energy excitations which entail disassociation of a bipolaron into two singly self-trapped electrons or vice versa.

Consider now an example of a thermodynamically inaccessible correlated state which could produce the desired functional dependence in χ'' . For this purpose assume a smooth density of electron pairing states in the neighbourhood of the 'Fermi level' and suppose that a field induced hop of the polarisable species introduces a coupling between these states which is largely independent of energy. In this instance one will expect an ω^{n-1} dependence in χ'' from very low energy excitations that involve the transfer of an electron pair from an occupied to initially unoccupied pairing centre. This behaviour, however, will be completely smeared out at experimental temperatures unless we suppose that the energy barriers between pairing states is sufficient to prevent thermally assisted tunnelling. Thus the system at finite temperature is presumed locked into a metastable state and this could happen for large enough negative U. This case is complementary to our previous examples where it is supposed that each TM has equal probability of being in either of its two states. Note that the possibility of low lying excitations which are thermodynamically inaccessible over at least the time of a specific heat measurement has already been pointed out¹⁵ and in amorphous materials metastable states may persist almost indefinitely. Furthermore in this metastable regime the response of the system should depend on its history.

Although in this section we have considered primarily amorphous systems where one expects an appreciable number of weaker/stronger bonds, lone pairs, and so on, to be present giving rise to the correlated states, it is also reasonable to expect that such low-lying excitations occur and are important in more nearly crystalline solids where, for example, the remaining pairing centres in these materials could partially pin the Fermi level in their vicinity. Another relevant point is the probable

presence of an appreciable density of correlated states at various interfaces such as oxide-semiconductor²⁹, and metal-semiconductor³⁰. Thus, although interface or contact effects are usually ignored, we expect that such systems should also exhibit a dielectric loss obeying the 'universal law' and a systematic study of the details could provide a powerful probe of the interfacial structure, so important in device physics.

Low frequency IR divergent dielectric response

Having argued that dielectrics with diverse interaction types should have invariably some very low frequency excitations that contribute a time dependence of the form t^{-n} at large t to some correlation function, we examine further the properties of the dielectric response function. Here we focus on the behaviour of $\chi''(\omega)$ starting several decades above any associated loss peak that may exist, since this is the region where the universal ω^{n-1} dependence is found (see Fig. 1). In doing this we do not exclude those dielectrics which have associated with them the presence of charge carriers of an electronic or an ionic nature. In this regime the dielectric loss is simply related to the probability of exciting the correlated states and is given by $\chi''(\omega) \propto \int_{-\infty}^{+\infty} dt e^{i\omega t} e^{-\phi(t)}$ where $\phi(t)$ describes the time response of the correlated states due to the sudden potential, V . The function $\phi(t)$ has been obtained many times^{7,8} and in our notation takes the form $\phi(t) \equiv \int_0^{E_c} V^2 N(E) (1 - e^{-iEt}) (dE/E^2)$. The existence of some class of correlated states in the dielectric with $V^2 N(E) \equiv nE$ produces an IR divergence in the number of low energy correlated states excited and for large E_c , $\phi(t)$ can be approximated by $n \ln(iE_c t)$. Putting this result back into the expression for χ'' leads immediately to the universal law $\chi''(\omega) \propto \omega^{(n-1)}$. Note that the loss peak observed in dipolar solids before the universal response can be accommodated as well within the present framework and will be discussed elsewhere. In addition, dielectrics with hopping electronic or ionic charge carriers would be expected to show a strong low-frequency dispersion, in accordance with experimental evidence.

Conclusions

We have arrived at a fundamental mechanism for the universal dependence of the dielectric loss in nearly all dielectrics at frequencies above any loss peak that may be present and the mechanism is operative independent of the physical structure and chemical bonding in the materials. We emphasise the importance here of not only the identification of the Curie-von Schweidler law as an IR divergence phenomenon but also the subtle task of finding the correlated state excitations that are responsible for it.

The entire empirically determined range of values of the exponent, $0 < n < 1$, can be accounted for in the present theory depending on the values of the exciting potential V arising from the transitions of the charged species and of the density of states of correlated state excitations. The appearance of the limiting form of the Debye response may be expected in situations where V or $N(E)$ are negligible. Our approach then produces a general scheme for classification of dielectrics based on the type of dominant correlations and correlated states they render. In particular, a discussion of correlated states associated with electron-electron, electron-lattice, ion-ion correlations as well as various subgrouping have been given. There may still be other types of correlations leading to correlated states with the same properties. In any event, dielectric loss should provide a powerful probe into details of the correlated states of the type we have considered here. We acknowledge financial support from the NSF and SRC.

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Correlation of the Eemian (interglacial) Stage and the deep-sea oxygen-isotope stratigraphy

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A complete interglacial sequence in coastal marine sediments in western Norway is correlated with the Eemian Stage by means of pollen stratigraphy, and with deep-sea cores by means of marine fossils. The Eemian is correlated with isotope stage 5e.

THE correlation of deep-sea and continental records is one of the most important problems in Quaternary stratigraphy. Because nearly all cores from the deep-sea floor can be correlated using the $^{18}\text{O}/^{16}\text{O}$ record^{1,2}, oxygen-isotope stratigraphy is also the most valuable method of correlation with the continents. The general principles in the correlation of glacial/interglacial periods with oxygen-isotope stages are known¹. However, the correlation of real stratigraphical units defined on land, and the oxygen-isotope stages remains problematic beyond the range of ^{14}C dating.

Here we correlate the Eemian Stage of North-west Europe with the oxygen-isotope stage 5e as follows: a complete interglacial sequence in coastal marine sediments in western Norway can be correlated with the Eemian by means of its pollen stratigraphy, and the marine fauna of the same section can be correlated with the biostratigraphy of cores from the Norwegian Sea³, in which the oxygen-isotope stratigraphy has also been established^{4,5}.

Shakleton⁶ first argued for the correlation of 5e with the Eemian, and his conclusion has been accepted by most scientists. Confirmation has, however, been lacking, and discussion of alternative correlations has continued. Off the Pacific coast of North America the correlation of 5e with the forest vegetation period of the last interglacial has been demonstrated⁷.

Definition and stratigraphical position of the Eemian

The Eem interglacial is now generally accepted as a chronostratigraphical unit with stage rank: the Eemian Stage⁸⁻¹⁰. The type locality is along the river Eem, near Amersfoort, Netherlands¹¹. The lower boundary is defined as where a subarctic park landscape at the type locality is replaced with a closed forest and

the upper boundary is placed where the forest at the type locality is replaced by a more open vegetation.

The Eemian in North-west Europe is clearly recognisable by its typical pollen stratigraphy^{12,13}. This approach could create confusion if the vegetational development during two interglacials were very similar. However, this does not seem to be a serious problem for the Eemian.

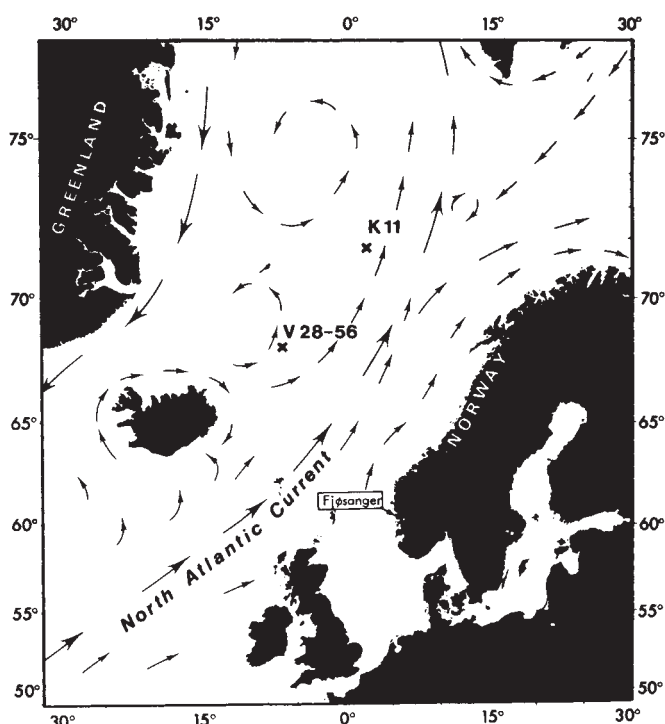


Fig. 1 Map of the Norwegian Sea and surrounding areas. The location of Fjøsanger and the cores mentioned in the text are indicated. The oceanic surface currents are also shown.

where. Two till horizons are located at the base of the sequence. Above these follows a sequence of marine sediments (beds N-E, Fig. 2), deposited in a fiord at depths of between 10 and 50 m during an interglacial and the earliest part of the following glacial period. The sediments consist of silt, sand and gravel (Fig. 2). However, even the coarse-grained sediments have a matrix of clay and silt, and both the benthic foraminifera and the pollen stratigraphy support an interpretation of continuous deposition of the clay-silt fraction throughout the marine sequence. The section is capped by a till.

We (in preparation) have defined the Fjøsangerian Stage with a lower boundary at the base of bed N (Fig. 2) and an upper boundary between beds I and H. The latter is also the lower boundary for the Hordalandian Stage.

The main criterion for the correlation of the Fjøsangerian Stage with the Eemian is the pollen stratigraphy (Fig. 2), the most significant features of which are: (1) the very distinct successional phases. (2) Early immigration of *Quercus*. (3) Development of a dense *Quercus-Corylus* forest, with very sparse occurrence of other QM-constituents. (4) *Alnus* increased at the same level as *Corylus*. (5) Late immigration of *Picea*, with a peak very late in the interglacial. The only main feature of the Eemian which is lacking is the *Carpinus* phase, which is almost certainly due to the northerly latitude of the Fjøsanger locality. The vegetation during the deposition of gravel I, indicates a mean summer temperature slightly higher than at present (Fig. 2).

At the type locality, the lower boundary for the Eemian Stage is placed at a horizon when a closed forest was established¹². By comparison with the Holocene vegetational development it may be assumed that the spread of *Betula* woodland at Fjøsanger took place slightly later than at the type locality in the Netherlands. Actually, Fjøsanger was still covered by ice at the Weichselian-Holocene boundary. Bearing this in mind, we have placed the lower boundary for the Eemian immediately after the deglaciation of the site, and we assume that the error in this time correlation should not exceed some few hundred years.

We have correlated the Eemian-Weichselian boundary with the Fjøsangerian-Hordalandian boundary where the NAP-pollen increases from ~5% to 30%. This clearly represents a complete deforestation of the area, and we assume that all tree pollen types above this level, except possibly some *Betula*, are redeposited.

The molluscs and benthic foraminifera lead to very similar conclusions, and therefore only the mollusc diagram is presented here (Fig. 2). The fauna indicates cold polar water in the lower part of the Eemian (silt M, Fig. 2) and thereafter a rapid increase in temperature due to the appearance of the Atlantic Current. In beds I and J the fauna indicates warmer water than along the coast today, and also species not known from Norwegian waters were (for example, *Parvicardium papillosum*). As some of the mollusc specimens are only referred to bed I, and the benthic foraminifera indicate a shallowing during the deposition of the upper part of this bed, it is difficult to detect a possible fall in temperature during the deposition of the upper part of bed I, but this must in any case have been very modest. From the deposition of bed I to that of bed H a drastic fall in temperature occurred, but the influence of the Atlantic Current is still detectable. In silt G, a polar fauna dominated, and during the deposition of this bed, glaciers were calving in the fiord. The fauna in gravel F indicates that the glaciers had retreated and this bed is assumed to correspond to an interstadial. Silt E represents another glaciomarine facies, above which the till follows. Whether or not a hiatus exists below the till is impossible to determine.

Three features of the Fjøsanger section are of major importance for correlation with the Norwegian Sea stratigraphy: (1) an oceanic circulation pattern similar to that of the present was established in the lower part of the Eemian. (2) During most of the Eemian, coastal waters were as warm, or warmer, than at present. (3) A drastic cooling of the sea water occurred at the Eemian-Weichselian boundary.

Correlation with the deep-sea record

Kellogg^{3,19} studied cores from the Norwegian Sea. Mainly by means of two faunal indices (Fig. 3), he has shown that only once during the past 450,000 yr has the surface water of the Norwegian Sea been as warm, or warmer, than during the Holocene.

The $^{18}\text{O}/^{16}\text{O}$ ratio has been measured in two of the cores from the Norwegian Sea, and one core from the Denmark Strait^{4,5}. The younger oxygen-isotope stages used in the world ocean stratigraphy were clearly recognised in these cores (Fig. 3).

The dominant factor determining the surface temperature of the sea both in the eastern Norwegian Sea and along the coast of Norway is the Atlantic Current. From a theoretical point of view, it is clear that faunas from the Norwegian coast, and the surface of the eastern Norwegian Sea, can be correlated by means of their temperature requirements. This is in accordance with knowledge of the present-day zoogeography, and also with geological correlations of the major change in the current pattern 10,000–13,000 yr ago^{20–23}.

The 'warm pulse' described by Kellogg can therefore be unambiguously correlated with the warm-water phase at Fjøsanger. The warm pulse in the Norwegian Sea appears at the same level as the oxygen-isotope stage 5e. As the warm-water phase at Fjøsanger occurred within the Eemian, the Eemian Stage can be correlated with the oxygen-isotope stage 5e.

Concerning a more precise correlation of the boundaries, the 6–5e boundary in the cores V 28–56 and K-11 (Fig. 3) is

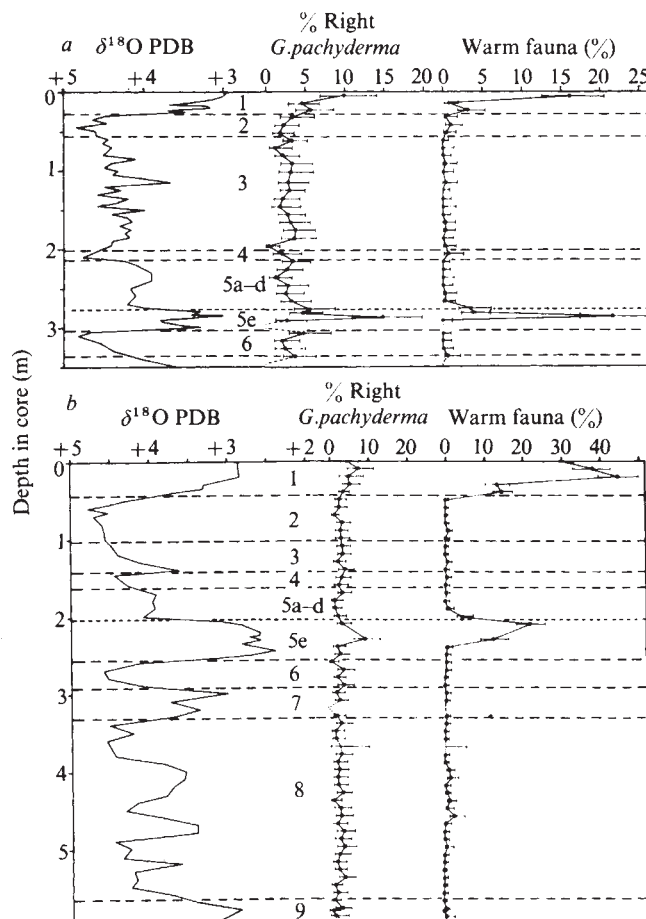


Fig. 3 The stratigraphy of two Norwegian-Sea cores (Fig. 1): a, K-11; b, V 28–56; according to Kellogg, Duplessy and Shackleton⁴. The curves represent (from the left): the $^{18}\text{O}/^{16}\text{O}$ curve; the oxygen-isotope stages (numbers); % right-coiling species (warm) of *Globigerina pachyderma*; and % warm-water foraminifera species.

significantly below the warm pulse. At Fjøsanger there is also a cold fauna in the lower Eemian. We have deduced, however, a high sedimentation rate for this part of the stratigraphy, and thus a short time period. This would indicate that the lower boundary for the Eemian is slightly above the 6–5e boundary.

In the cores from the Norwegian Sea (Fig. 3), the upper boundary of the warm pulse is very close to the 5e–5d boundary. At Fjøsanger the dramatic cooling of the seawater also occurred at the Eemian–Weichselian boundary, indicating that the Eemian–Weichselian boundary is nearly synchronous with the 5e–5d boundary.

These correlations are also supported by sea-level data from the North Sea area, for which Zagwijn²⁴ has given the best data. A major part of the transgression took place within the Eemian, indicating that the Eemian started long before the peak of the $^{18}\text{O}/^{16}\text{O}$ curve. Around the Eemian–Weichselian boundary, Zagwijn²⁴ found a drop in sea level of 32 m, of which half took place within the Eemian. Compared with the $^{18}\text{O}/^{16}\text{O}$ sea-level curve derived by Shackleton and Opdyke¹, this suggests that the Eemian–Weichselian and the 5e–5d boundaries are synchronous.

The sea-level data are also a very strong argument, independent of the main theme of this paper, that the Eemian should be correlated with 5e, and not a larger, or other part of stage 5.

The onset of the last glaciation

The forests at Fjøsanger during the later part of the Eemian indicate a climate which excluded the growth of any large ice-masses in Scandinavia. The drop in sea level during the Eemian thus indicates that the growth of ice sheets started earlier in North America and/or Antarctica than in Scandinavia.

At Fjøsanger the glaciomarine silt G (Fig. 2) confirms that glaciers approached Bergen early in the Weichselian. From the present day topography and climate, one can conclude that these first glaciers descended from mountains in the vicinity, and were not outlets from an inland-ice. These glaciers retreated at least once (bed F, Fig. 2) before Fjøsanger was covered by ice.

The vegetational succession during the upper part of the Eemian indicates, in our opinion, a climatic deterioration. However, the drastic fall in temperature at Fjøsanger occurred simultaneously in the sea and on land at the Eemian–Weichselian boundary. There can be no doubt that the cause of the sudden cooling was a weakening of the flow of the Atlantic Current into the Norwegian Sea. If the Atlantic Current was

'switched off' the sequence described would result, whether or not this event was preceded by, or caused by, a climatic deterioration.

The radiometric age of the Eemian

From the ^{14}C isotope enrichment method, the age of the early Weichselian interstadials has been estimated as 55,000–70,000 yr BP²⁵, and the Eemian is commonly assumed to be slightly older.

Another approach is correlation with tropical coral reef terraces, dated with the U-series methods. Shackleton and Matthews²⁶ showed that the oxygen-isotope stage 5e can be correlated with the Barbados III terrace, dated to 125,000 yr BP. Accepting the correlation of 5e with the Eemian, this age should also apply to the Eemian.

There is a significant discrepancy in the results from the two methods. At present we find the U-series ages most probable.

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Nucleotide sequence of a region of human mitochondrial DNA containing the precisely identified origin of replication

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A fragment of HeLa cell mitochondrial DNA containing the origin of replication has been sequenced. The precise position of the origin in this sequence has been identified by determining the nucleotide order in the 5'-end proximal portion of the heavy strand initiation fragment (7S DNA), and by aligning the two sequences.

MITOCHONDRIAL DNA synthesis in animal cells has been shown to have very distinctive features. In mouse L cells, where this process has been studied in most detail, DNA replication starts at a site located near the 5'-end of the 12S rRNA coding sequences with the displacement synthesis of a daughter H-strand, which grows in the direction away from the rRNA genes. When the nascent H-strand is at least two-thirds complete, synthesis of the complementary L-strand DNA begins at a point

The diagram illustrates the Hpa II restriction site and its recognition sequence. The top part shows a linear DNA sequence with a D-loop and a D-loop. The bottom part shows a circular DNA sequence with a D-loop and a D-loop. The diagram is labeled with various numbers and letters, including 12S, 4S, 3, 8, 17, 10, 2, Hpa II, Hae III, and D-loop.

Equally unique to mit-DNA of most animal cells investigated so far is the presence, in a substantial portion of the molecules, of a displacement loop (D-loop) near the origin of replication. This loop results from the synthesis of a short segment of H-strand DNA which displaces the parental H-strand⁷. These single-strand segments of DNA (referred to as 7S DNA because of their sedimentation coefficient in sucrose gradients) have recently been shown to exist as discrete size classes ranging between 500 and 700 nucleotides depending on the cell type. In mouse L cells, most of the 7S DNA molecules have a common 5'-end with a variability in length at the 3'-end⁸, whereas, in human cells, the variability has been reported to be at the 5'-end⁹ or at both ends⁸. In L cells there also seems to be a microheterogeneity at the 5'-end of each predominant size class⁸. Recent studies have shown that the 7S DNA is unstable, with a half life of about 1 h in L cells¹⁰ and 2.8 h in human KB cells (D. Bogenhagen and D. Clayton, personal communication). Therefore, if 7S DNA is a primer for DNA replication, only a small fraction of the 7S DNA molecules can serve this role, as mit-DNA is known to be stable. The true function(s) of 7S DNA is still unknown. It may reflect a need for the cell to be able to recognise the origin or to continuously expose the single-stranded parental H-strand for initiation of transcription. Conceivably also, the D-loop may be involved in the recently discovered interaction between the mitochondrial inner membrane and the origin of mitochondrial DNA replication¹¹.

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$\Delta 8b^{Hae}$ (~200 base pairs), and that the origin is contained in $\Delta 8b^{Hae}$ (ref. 13). This information, summarised in Fig. 1, has thus provided the foundation for the sequencing work described here.

Sequence analysis of the region of mit-DNA containing the origin of replication

The approach followed here to sequence the region of mit-DNA around the origin of replication involved isolating the restriction fragment $\Delta 8b^{Hae}$, labelling the 5'-ends with [γ - 32 P]ATP and T4 polynucleotide kinase, separating the strands and sequencing both strands by the technique of Maxam and Gilbert¹⁶. As the fragment $\Delta 8b^{Hae}$ was known to be around 200 nucleotides long, we hoped that by ordering more than 100 nucleotides from each end, we could sequence the entire fragment with a substantial sequence overlap. Fragment $\Delta 8b^{Hae}$ was isolated by gel electrophoresis, eluted and subjected to two kinds of kinase reactions: (1) the exchange reaction^{17,18}, which does not require prior dephosphorylation of the 5'-terminus of the DNA, and (2) removal of the 5'-phosphate with bacterial alkaline phosphatase and then treatment with polynucleotide kinase¹⁶. Figure 2 shows the results of the kinase reactions on strand separation on a polyacrylamide gel. Significantly, in the DNA labelled by the exchange reaction, the faster migrating band contained only 15% as much radioactivity as the slower moving band, whereas DNA labelled after dephosphorylation revealed a faster migrating band labelled to the extent of about 65% of the slower band. As the incorporation into the slower migrating band was similar in the material labelled by the two procedures, in the present work, the DNA was routinely dephosphorylated before treatment with polynucleotide kinase.

After elution of the material in the two labelled DNA bands, aliquots of each sample were hybridised to an excess of either the heavy or the light strand of HeLa cell mit-DNA, incubated with S_1 nuclease, and the acid precipitability measured. The DNA from the faster migrating band hybridised to the extent of about 56% of the input c.p.m. to the H-strand and of 2% to the L-strand, whereas about 42% of the input c.p.m. in the material from the slower migrating band hybridised to the L-strand and 0.6% to the H-strand. The lack of complete hybridisation to mit-DNA of the two separated $\Delta 8b$ strands is presumably due to the occurrence of some breaking at the ends of the hybrids, with the S_1 nuclease cleaving off a portion (40–50%) of the 5'-labelled nucleotides. On the basis of these data, we have designated the faster migrating DNA strand, $\Delta 8b^{Hae}(H)$, and the slower migrating DNA strand, $\Delta 8b^{Hae}(L)$. It is not clear why the dephosphorylated and kinased $\Delta 8b^{Hae}(H)$ contained a lower amount of radioactivity than $\Delta 8b^{Hae}(L)$ (varying, in different experiments between 75% and 31%). This difference is probably due to incomplete dephosphorylation, or to renaturation and subsequent lower incorporation during kinase treatment at the flush end created by the Hae III enzyme relative to the staggered 5'-end produced by Hpa II, or to some other feature, such as secondary structure.

Sequence analysis of the labelled DNA strands was carried out by the Maxam and Gilbert procedure¹⁶, using the G>A, A>C, C+T and C reactions. The sequence of nucleotides 2 to 25 was determined by fractionating the cleavage products on a 25% polyacrylamide–7 M urea gel; nucleotides 26 to 148 for $\Delta 8b^{Hae}(L)$ and nucleotides 25 to 123 for $\Delta 8b^{Hae}(H)$ were identified by fractionating the reaction products on 10% polyacrylamide–7 M urea gels (Fig. 3). The total length of the fragment was found to be 221 nucleotides, and thus it was possible to obtain an approximately 50 nucleotide overlap.

To complete the sequence, the end-labelled separated strands were digested exhaustively with endonuclease P_1 , which generates 5'-mononucleotides²¹, and the products were

Table 1 5'-terminal nucleotide analysis of $\Delta 8b^{Hae}(L)$, $\Delta 8b^{Hae}(H)$ and 7S DNA

	% of total 32 P c.p.m.*							
	dAMP	dCMP	dGMP	dTMP	AMP	CMP	GMP	UMP
$\Delta 8b^{Hae}(L)$	0.15	98.6	0.61	0.68				
$\Delta 8b^{Hae}(H)$	0.43	91.8	5.2	2.6				
7S DNA								
After isolation								
Expt 1	0.6	1.0	3.3	94.2	0.1	ND	ND	ND
Expt 2	1.0	1.4	4.4	93.1	ND	ND	ND	ND
In situ	2	5	3	89	ND	1	1	ND

5'-end-labelled $\Delta 8b^{Hae}(L)$ and $\Delta 8b^{Hae}(H)$ were dissolved in 15 μ l 50 mM ammonium acetate, pH 5.4, and digested with 1 μ g P_1 endonuclease from *Penicillium citrinum* for 1 h at 37 °C²¹. After addition of 10 nmol each of 5'-dAMP, dCMP, dGMP and dTMP, the mixture was applied to a PEI cellulose thin layer strip. After washing the strip in methanol, one-dimensional thin layer chromatography was carried out using 1 M acetic acid for 2 cm and then 1 M acetic acid–3 M LiCl(9:1) for 16 cm²². 7S DNA, with 15 μ g of yeast tRNA carrier, was dissolved in 15 μ l 50 mM ammonium acetate, pH 5.4. The mixture was digested with 2.5 μ g of P_1 at 37 °C for 3 h. After addition of marker 5'-dNMPs, the digest was spotted onto a PEI cellulose plate and run in the first dimension as above. The plate was washed successively with Tris–methanol and methanol, and then run in a second dimension for 16 cm using 6 g Na₂B₄O₇ · 10H₂O, 3gH₃BO₃ and 25 ml ethylene glycol in 70 ml water²². All thin layer plates were examined by autoradiography sensitive enough to detect 1% of the input radioactivity (3 d.p.m. in a 1-mm² circle), and then the marker spots were visualised with a short-wave UV lamp and the spots cut out. They were eluted quantitatively with 1 ml of 0.02 M Tris–HCl, pH 7.4, 0.7 M MgCl₂ and counted in 10 ml of a Triton–xylene based scintillation fluid. ND, not detectable.

* The values refer to the radioactivity which migrated from the origin. The % of the input radioactivity recovered from the origin was, respectively, 0.5 and 4.9% for $\Delta 8b^{Hae}(L)$ and $\Delta 8b^{Hae}(H)$, not detectable and 1.2% for isolated 7S DNA (experiments 1 and 2, respectively) and 2.0% for the *in situ* labelled 7S DNA.

separated by one-dimensional chromatography on thin layer polyethylenimine strips. The results shown in Table 1 indicate that the 5'-terminal nucleotide of both the H and L strands is dCMP, as expected for the Hpa II and Hae III cutting sites. The complete sequence of the fragment $\Delta 8b^{Hae}$ is shown in Fig. 4.

Isolation and characterisation of 7S DNA

To precisely localise the origin of mitochondrial DNA replication in the $\Delta 8b^{Hae}$ fragment, the approach followed was to sequence the 5'-end portion of the 7S DNA and to align this sequence with that of $\Delta 8b^{Hae}$. Previous reports of 7S DNA size heterogeneity in human, mouse and other animal cells and also of an apparent 7S DNA 5'-end microheterogeneity in mouse L cells required that HeLa cell 7S DNA be first characterised in its size and 5'-termini.

Isolation of 7S DNA was carried out by the procedure of Kasamatsu *et al.*⁷, in which the 7S DNA molecules are released from closed circular mit-DNA by brief heat treatment and then separated by sucrose gradient centrifugation. The material in the 7S DNA peak was further purified from RNA contaminants by RNase digestion or nitrocellulose chromatography²³, and then isolated by polyacrylamide or agarose gel electrophoresis in denaturing conditions.

Figure 5 illustrates the purification procedures used. Slot 2 shows the pattern obtained by electrophoresing through a 4% polyacrylamide–7 M urea gel a sample of sucrose gradient-isolated 7S DNA labelled *in vitro* at the 5'-end with [γ - 32 P]ATP and T4 polynucleotide kinase. A prominent band (indicated as 1) is seen migrating at a position corresponding to a length of about 680 nucleotides (estimated by comparison with marker HeLa cell Hpa II fragments). A fainter band (indicated as 2), corresponding to a size of about 630 nucleotides, can also be seen. The further purification achieved by nitrocellulose chromatography is shown in slot 3, where the discrete and heterogeneous components visible in the lower part of the gel and presumably arising from RNA contaminants have been

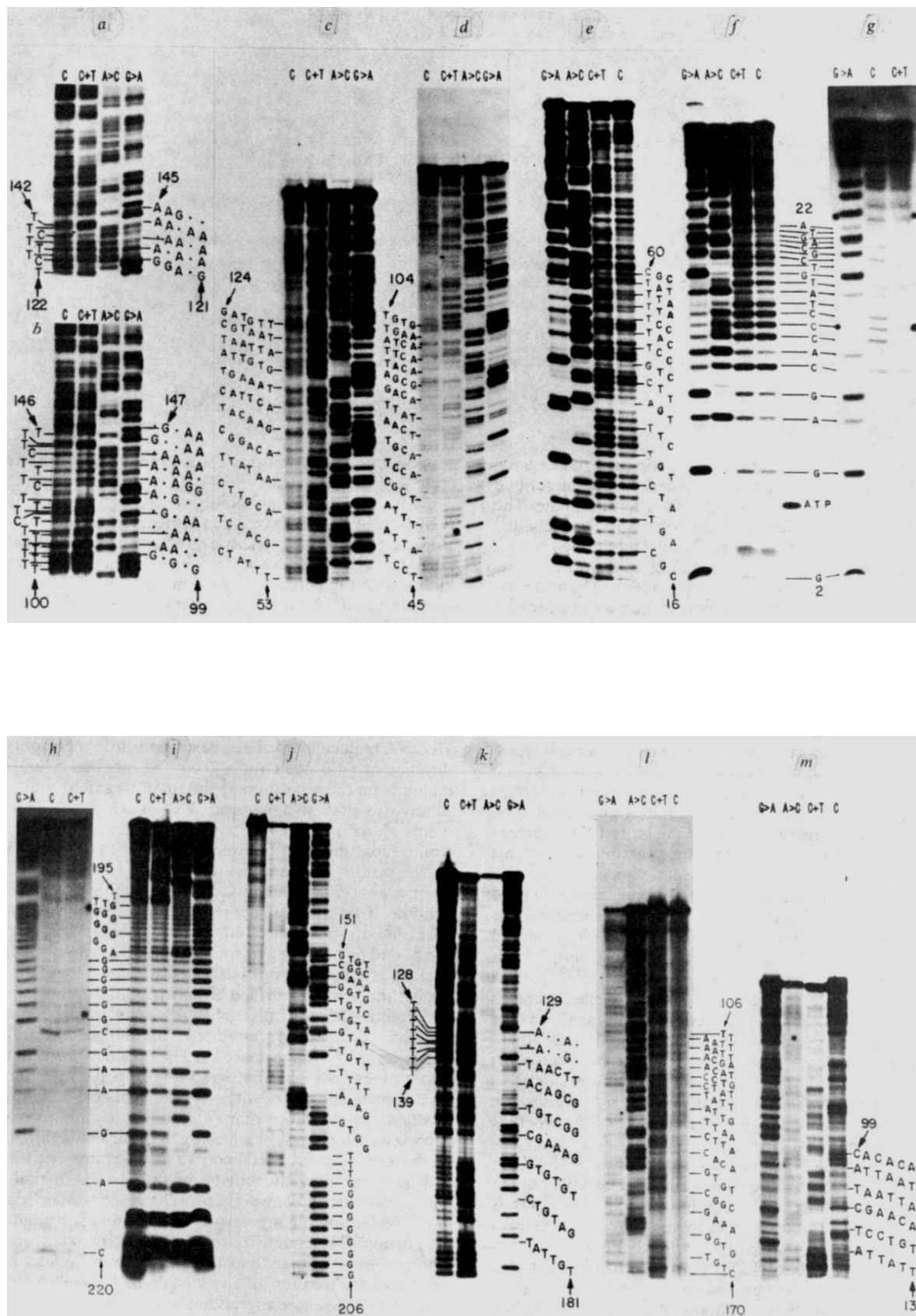


Fig. 3 Representative autoradiographs of sequencing gels of $\Delta 8b^{Hae(L)}$ (a-g) and $\Delta 8b^{Hae(H)}$ (h-m). The DNA strands, labelled at the 5'-end with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and polynucleotide kinase after alkaline phosphatase treatment (see text), were sequenced using the chemical methods of Maxam and Gilbert. The sequence reactions used and the times of reaction for the base modification step were G>A (10 min), A>C (10 min), G + T (15 min) and C (15 min). The degradation products were electrophoresed at 1,000 V for a, 15.5; b, 13; m, 12; c, 1, 8.5; d, k, 7.25; f-i, 7 and e, j, 4 h on either 10% polyacrylamide (1:20 bisacrylamide)-7 M urea gels (a-e, j-m) or 25% polyacrylamide (1:30 bisacrylamide)-7 M urea gels (f-i) (40x30x0.15 cm) in 0.05 M Tris-borate, pH 8.3, 0.001 M EDTA, that had been pre-electrophoresed for 2 and 3 h, respectively, at 900 V. The gels were wrapped with Saran wrap and exposed at -70 °C using pre-fogged Kodak X-Omat XR-5 film with a DuPont Cronex Lightning-Plus intensifying screen.



Fig. 4 Nucleotide sequence of the fragment $\Delta 8b^{Hae}$ and of the 5'-terminal segment of 7S DNA.

eliminated. Slot 4 shows the electrophoretic pattern obtained with 7S DNA prepared, using the isolation procedure described above, from cells labelled *in vivo* with ^{32}P -orthophosphate. The pattern observed is very similar to that of the *in vitro* labelled material. Here, in addition to the two above mentioned bands, there is also a third faint band migrating at a position corresponding to a size of about 590 nucleotides. These observations on *in vivo* labelled material indicate that the appearance of the minor 7S DNA components is not due to artefacts introduced by the *in vitro* labelling conditions.

Comparison of the electrophoretic patterns of sucrose gradient-purified 7S DNA with those published by Brown *et al.*⁹ and Gillum and Clayton⁸ suggests that the major and the minor bands observed here correspond to the three major forms of 7S DNA detected by these authors in several human cell types. However, the relative intensities of the bands observed by the cited authors and those shown here are strikingly different. Instead of three components in approximately equal amounts, our material shows a preponderance of the largest, 680 nucleotide long, component. To exclude the possibility that this difference resulted from losses or degradation occurring during purification of the 7S DNA, the *in situ* labelling technique used by Gillum and Clayton⁸ was followed. In this procedure, the 7S DNA is labelled without prior separation from closed circular mit-DNA. In Fig. 5, slot 5, where the results of this experiment are presented, the electrophoretic pattern seen is very similar to that of the *in vitro* labelled samples with the 680 nucleotide long component again being strongly predominant. Densitometric analysis of these autoradiograms indicate that this component represents 70–80% of the total 7S DNA in preparations labelled *in situ*, and more than 95% in preparations labelled after sucrose gradient isolation, assuming equal efficiency of *in vitro* labelling of the various components.

The fact that the relative intensities of the bands produced by the two labelling methods were comparable did not rule out the possibility that the difference in mobility between the 7S DNA components might be due to some factor other than heterogeneity in length. However, treatment with RNase (Fig. 5, slot 4) or pronase (in the presence of SDS) before electrophoresis failed to alter the observed number, relative abundance, or electrophoretic mobility of these components, thereby excluding any possible role of a covalently linked RNA primer or of DNA-associated protein in generating this phenomenon. In addition, when the *in situ* labelled component 2 and the *in vitro* labelled component 1 were isolated from the urea gel, heat denatured and re-run through a second urea gel in the same conditions (Fig. 5, slots 6 and 7, respectively), or through an agarose- CH_3HgOH gel²⁶ (not shown), they maintained the same relative electrophoretic mobility. The above results

support the conclusion that the multiple bands arise from discrete length differences.

To investigate the possibility of 5'-end microheterogeneity (which would not have been detected by the above electrophoretic analysis), sucrose-gradient-isolated 7S DNA was labelled at the 5'-end with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and T4 polynucleotide kinase and subjected to digestion with the *Hpa*II restriction enzyme. (*Hpa*II is known to cleave single-stranded DNA²⁷, and previous experiments in this laboratory had indicated that the *Hpa*II site present in the single stranded portion of the D-loop (which is equivalent in sequences to the 7S DNA) is cut at a low frequency in standard digestion conditions¹³.) The digest was analysed on a 7.5% polyacrylamide-7M urea gel; the results obtained (not shown) indicated that a small percentage of the 7S DNA molecules had been cut at a position corresponding to a distance of ~86 nucleotides from the labelled 5'-end (as estimated from the migration of the small fragment thus produced relative to that of denatured ΦX174 RF DNA *Hae*III fragments)²⁸. As the resolving power of the gel electrophoresis used would have shown a difference of 1 or 2 bases in length, the above results indicate that the 5'-end of the main 7S DNA component occurs at a unique position within the mitochondrial genome. To obtain further evidence on this question, a 5'-end base analysis was carried out. 7S DNA was dephosphorylated at the 5'-end by alkaline phosphatase either *in situ* or *in vitro* after sucrose gradient isolation, labelled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and polynucleotide kinase, purified by nitrocellulose chromatography and electrophoresed through a 4% acrylamide-7M urea gel. The main component was eluted from the gel and subjected to digestion with endonuclease P_1 . The products were analysed by two-dimensional chromatography on polyethylenimine thin layer plates, and the results are given in Table 1. In the *in vitro* labelled sample, more than 90% of the counts recovered are represented by dTMP, confirming the conclusion that the 5'-end of the predominant HeLa cell 7S DNA component is unique. Furthermore, no radioactivity, or only traces of radioactivity, were detected in ribonucleotides. The results obtained for the *in situ* labelled sample are very similar, although a slightly larger proportion of radioactivity seems to be associated with deoxyribonucleotides other than dTMP; this may reflect the lower degree of purification of *in situ* labelled 7S DNA due to the omission of the sucrose gradient purification step.

Sequence analysis of 7S DNA

The main 7S DNA component was subjected to alkaline phosphatase treatment and the kinase reaction either *in situ* or after sucrose gradient purification, passed through a nitrocellulose column and then run on a polyacrylamide-urea gel. After elution of the 7S DNA from the gel, it was submitted to the Maxam-Gilbert sequencing reactions.

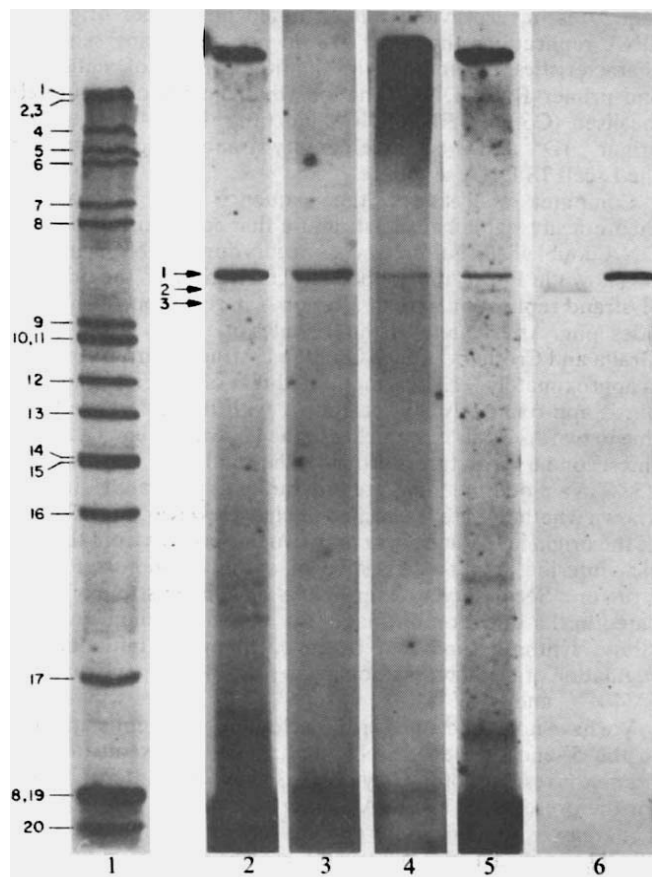


Fig. 5 Autoradiographs, after electrophoresis through 4% polyacrylamide-7 M urea gels, of HeLa cell 7S DNA labelled *in vitro* or *in vivo* and purified in different ways. All samples, in 0.001 M Tris-HCl, pH 7.4, 0.001 M EDTA, were adjusted to 6 M urea, heated at 100 °C for 1 min and loaded on a 4% polyacrylamide (1:30 bisacrylamide)-7 M urea slab gel. Electrophoresis was carried out at 5 V cm⁻¹ for 15 h. *Slot 1*: *Hpa*II digest of HeLa cell mit-DNA labelled *in vitro* with [α -³²P]dNTP and *E. coli* DNA polymerase I¹². *Slots 2 and 3*: 7S DNA was released from mit-DNA by brief heating, isolated by sucrose gradient centrifugation⁷, and labelled *in vitro* at the 5'-end as follows: A sample of ~0.2 pmol of 7S DNA (estimated by assuming a proportion of about 10% D-loop containing molecules in HeLa cell mit-DNA preparations²⁴, and a complete recovery of the isolated 7S DNA) was dephosphorylated and 5'-end labelled as described in Fig. 2 legend. After ethanol precipitation, the pellet was dissolved in 0.001 M Tris-HCl, pH 7.4, 0.001 M EDTA. A portion of the sample was analysed directly (slot 2) and a portion was further purified by nitrocellulose (NC) chromatography. For this the sample (50 µl) was adjusted to 0.5 M KCl and passed through a 1.5 x 0.3 cm nitrocellulose column (Hercules, prepared as described by Boezi and Armstrong²⁵) equilibrated with 0.5 M KCl, 0.01 M Tris-HCl, pH 7.4. The column was washed with equilibration buffer to remove all the unbound radioactivity, then eluted with 0.001 M Tris-HCl, pH 7.4, 0.001 M EDTA (slot 3). *Slot 4*: 7S DNA from 1.5 x 10⁸ cells labelled *in vivo* with ³²P-orthophosphate¹² was isolated by sucrose gradient centrifugation and run on gel after treatment with pancreatic RNase (40 µg ml⁻¹, 10 min at 25 °C). *Slot 5*: 7S DNA labelled *in situ* following the procedure of Gillum and Clayton⁸: a sample of 10 µg mit-DNA in 100 µl 0.01 M Tris-HCl, pH 8.5, 0.01 M NaCl, was incubated with 0.05 µg calf intestine alkaline phosphatase (Boehringer Mannheim) for 1 h at 57 °C, and then labelled with [γ -³²P]ATP and T4 polynucleotide kinase. *Slots 6 and 7*: 7S DNA components 2 and 1, which had been labelled at the 5'-end *in situ* or after sucrose gradient isolation, respectively, then purified by nitrocellulose chromatography, and run through a 4% polyacrylamide-7 M urea gel, were eluted and rerun in the same conditions.

Figure 6 shows a sequencing gel of 5'-end labelled, sucrose gradient-purified 7S DNA. A sequence of around 25 nucleotides could be determined unambiguously. This sequence is identical to the portion of the sequence of $\Delta 8b^{Hae}$ from residue 87 to residue 63. This alignment allowed the positioning of the 5'-end of 7S DNA to correspond with residue 87 of $\Delta 8b^{Hae}$. Sequencing experiments using *in situ* labelled 7S DNA gave identical results²⁸.

Discussion

In the experiments reported here, we have precisely located the origin of HeLa cell mit-DNA synthesis in the *Hpa*II map of this DNA and examined the sequence surrounding it. The location of the 5'-end of the 7S DNA, determined by sequence analysis to be at 87 nucleotides from the *Hpa*II cutting site between fragments 8 and 17, is in excellent agreement with the 7S DNA priming experiments that indicated the origin to be at approximately 80 nucleotides from the *Hpa*II site, and with the experiments in which 5'-end labelled 7S DNA was cut with *Hpa*II giving a labelled DNA fragment about 86 nucleotides long. The size of $\Delta 8b^{Hae}$ is 221 nucleotide pairs, which is about 10% longer than the value that was earlier derived from its electrophoretic mobility in agarose gels relative to that of *Hpa*II fragments.

There are several interesting features of the $\Delta 8b^{Hae}$ sequence (Fig. 4), although their significance is unclear. Beginning at nucleotide 215 and proceeding clockwise (the clockwise direction is here defined as the direction of H-strand synthesis), there is a stretch of 17 G·C base pairs in a row with an A·T pair interruption at position 207. Furthermore, there is a strong purine bias on the H-strand. Nearby, from position 189 to 181, there is a stretch of 9 A·T base pairs in succession. There is another A·T base pair stretch running from position 137 to 128, and, contained within it, an 8 nucleotide true palindrome from

position 137 to 130. Closer to the origin is a 13 A·T base pair stretch which contains two true palindromes (113-104 and 116-105) and a sequence with a twofold axis of symmetry (115-104). Repeated sequences are relatively rare; the sequence 5' TTGTTAT 3' at nucleotides 140-134 is repeated 3' AACATA 5' at positions 182-176, and the sequence 5' GTGGAAA 3' is found at nucleotides 166-160 and at 193-187. The sequence

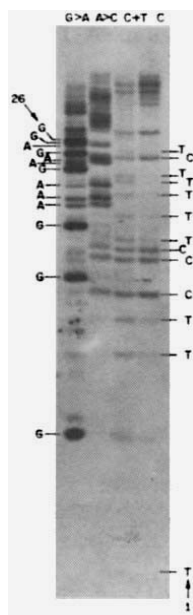


Fig. 6 Representative autoradiograph of 7S DNA sequencing gel. Sucrose gradient isolated 7S DNA was dephosphorylated and 5'-end labelled, as detailed in Fig. 2 legend, purified by nitrocellulose chromatography and then run through a 4% polyacrylamide-7 M urea gel. The main component (1) was eluted and subjected to the sequencing reactions G>A, A>C, C>T and C. Due to the low amount of radioactivity in the 7S DNA preparations, the base modification reactions were carried out for a time 10 times longer than used for the $\Delta 8b^{Hae}$ strands (legend to Fig. 3). This extended reaction time resulted in a decreased mobility of the products in the A>C reaction. The cleavage products were electrophoresed on a 25% polyacrylamide-7 M urea gel for 6.3 h.

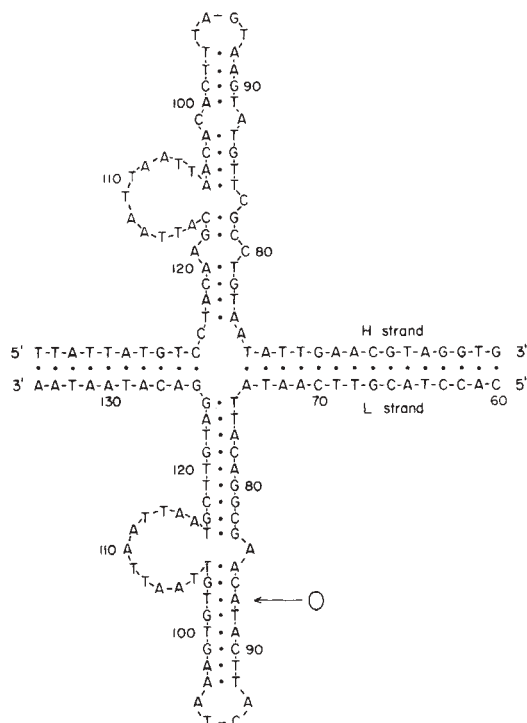


Fig. 7 Possible secondary structure of the region of fragment $\Delta 8b^{Hae}$ surrounding the origin of replication. The arrow (O) points to the nucleotide in the L-strand which is complementary to the 5'-end nucleotide of 7S DNA. The ΔG values of the L-strand and of the H-strand portions of the structure were calculated on the basis of the thermodynamic data for Watson-Crick nearest-neighbour sequences, loops, mismatches, and G·U pairs, as compiled for ribonucleic acids (see text). The free energies of base-pair addition were reduced by about 15% whenever the base-pair doublet included a G·C pair, in order to correct for the greater stability of the rG·rC pair as compared to dG·dC³⁴. In view of the many assumptions underlying these calculations, the ΔG values reported in the text have to be considered only as rough indicators of stability.

5'TATGATG 3', often found to be associated with promoter sites²⁹, is located at nucleotides 178 to 172 and might possibly be associated with an L-strand promoter site.

The origin itself is preceded, going in a clockwise direction, by a 29-nucleotide long A·T rich region that extends 3 nucleotides past the origin, is followed by a 6-nucleotide G·C rich region and then by a 10-nucleotide long A·T rich region. A·T rich

sequences have previously been noted near other origins of DNA replications; however, we do not detect any sequence characteristics in common with the other origins of replication and primer RNA-DNA junctions that have been accurately localised (ColE1³⁰ RNA-DNA junction, Φ X174 viral strand origin³¹, fd³² and G4 complementary strand³³ origins) nor with the L-cell 7S DNA sequence⁸.

Computer analysis of the sequence data indicates a theoretically stable hairpin structure that could form when the DNA double helix is unwound, as occurs during DNA synthesis. As shown in Fig. 7, this looped structure contains the origin of H-strand replication and an interior A·T rich loop, 12 nucleotides long. As calculated using the rules of Tinoco *et al.*^{35,36} and Gralla and Crothers³⁷, the ΔG of the L-strand hairpin structure is approximately $-13 \text{ kcal mol}^{-1}$ and that of the H-strand hairpin is approximately -5 kcal mol^{-1} , with the difference being due to two A·C pair mismatches in the H-strand stem. Although this secondary structure is probably thermodynamically stable at 25 °C in a moderate ionic strength buffer at neutral pH, it is not known whether it is ever formed *in vivo*. However, the presence of the origin in the hairpin at only two nucleotides from the A·T rich interior loop is suggestive of a functional role for this structure. Secondary structure loops have been strongly implicated in the initiation of phage fd³² and G4³³ complementary strand synthesis, and may be involved in the initiation and regulation of DNA replication in the mammalian papovaviruses SV40^{38,39} and BKV⁴⁰.

We have not detected any ribonucleotides covalently attached to the 5'-end of the 7S DNA that might represent an RNA primer or residues of it. Clayton *et al.*⁸ have presented evidence for the presence of an RNA primer in mouse L cell 7S DNA. The reason for this difference is unknown. In HeLa cell 7S DNA, we have also observed a greater homogeneity in size than previously reported in 7S DNA from other human cell types and other organisms. Nor have we detected the 5'-end microheterogeneity described in these systems. Our sequence analysis has not revealed any repetition of sequence or other structural features that might point to the existence of multiple initiation points, thereby explaining the existence of the multiple size classes. Instead, we think that the discrete size classes and the relative amounts of 7S DNA contained in each of them, the 5'-end microheterogeneity and the presence of RNA primer residues may reflect various rates or pathways of 7S DNA processing which could be dependent on cell type and/or physiological conditions.

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Upper limits for γ -ray bursts from primordial black holes

PAGE AND HAWKING¹ have predicted that primordial black holes of mass $\sim 10^{15}$ g will evaporate, reaching an explosive stage at a mass which depends on the nuclear processes occurring. For the composite particle model 10^{34} erg of γ rays will be produced at energies of 100–1,000 Mev. For the elementary particle model 10^{30} γ rays of energy 5×10^{12} eV are expected. We have already set upper limits^{2,3} for the rate of primordial black hole explosions in the Galaxy assuming the composite particle model. We report here upper limits from two other sets of data, assuming the elementary particle model and using the atmospheric Cerenkov technique as before.

In the first data set the Mt Hopkins 10-m reflector⁴ was used with a field of view of 1° . Approximately 112 h of observation were analysed. As the detector has an energy threshold of 10^{11} eV it will respond to 5×10^{12} eV primaries over a large area. Events were recorded on analogue magnetic tapes together with a standard frequency and time markers. They were analysed by reconstituting the standard frequency with a phase-locked loop, and after scaling, deriving a 0.1 s clock signal. The number of events recorded in each successive 0.1 s interval was determined with a mini-computer and a distribution derived. The characteristic signature of a γ -ray burst would be a group of individual showers occurring together within 0.1 s.

At five points in the data bursts were observed which were incompatible with random Poisson expectation. Four of these were very large and had durations of 2–11 s. They were probably local in character and due to man-made interference. The fifth event was just above the burst threshold (12 showers) and lasted 0.1 s. Without a distant coincident detector this event could not be ascribed to a cosmic origin. We therefore derived an upper limit at the 99% confidence level of 6.1 events in 112 h, or 477 events yr^{-1} .

The collection area of showers of 5×10^{12} eV was estimated at $5 \times 10^9 \text{ cm}^2$ and the solid angle was taken conservatively at the geometric value of $2.4 \times 10^{-4} \text{ sr}$. Twelve showers were required for a detectable burst, corresponding to 96 erg and giving a sensitivity of $1.9 \times 10^{-8} \text{ erg cm}^{-2}$. The maximum detectable distance r is then given by $4\pi r^2 = 8 \times 10^{30} / 1.9 \times 10^{-8} \text{ cm}^2$, hence $r = 5.7 \times 10^{18} \text{ cm} = 1.9 \text{ pc}$. The sensitive volume $V = \Omega r^3 / 3 = 5.5 \times 10^{-4} \text{ pc}^3$. The upper limit for the explosion rate is then 8.7×10^5 explosions $\text{pc}^{-3} \text{ yr}^{-1}$.

In the second data set⁵ two 1.5-m reflectors were used in coincidence aligned parallel to each other at varying zenith angles. The full field of view was 5° ; 78 h of observation were obtained. The normal counting rate was about 300 showers min^{-1} at the zenith. The energy threshold for the system was 3×10^{12} eV and the collection area for 5×10^{12} eV γ -rays estimated at $3 \times 10^9 \text{ cm}^2$. Events were recorded on analogue magnetic tape as before.

No groups of events were recorded beyond Poisson expectation. Seven events would be required for a statistically detectable burst, corresponding at 5×10^{12} eV to 56 erg and giving a sensitivity of $1.8 \times 10^{-8} \text{ erg cm}^{-2}$. The maximum detectable distance r is then given by $4\pi r^2 = 8 \times 10^{30} / 1.8 \times 10^{-8} \text{ cm}^2$ hence $r = 5.9 \times 10^{18} \text{ cm} = 2.0 \text{ pc}$. The sensitive volume $V = \Omega r^3 / 3 = 1.6 \times 10^{-2} \text{ pc}^3$. The upper limit at the 99% confidence level for zero bursts observed is 4.3 bursts in 78 h or 483 yr^{-1} .

The upper limit for the explosion rate is then 3.0×10^4 explosions $\text{pc}^{-3} \text{ yr}^{-1}$.

Using particle detectors at sea level with a higher energy threshold, which required some assumptions about the primordial black hole emission spectrum, Fegan *et al.*⁶ have found lower limits than those reported here. A further improvement in the Cerenkov method would require the use of one or more detectors with considerably larger collection area, and if possible, a wider field of view. A limit of about 1 explosion $\text{pc}^{-3} \text{ yr}^{-1}$ is set irrespective of the nuclear model assumed, by observations of the isotropic γ -ray spectrum¹. While the limit set with the Cerenkov technique assuming the composite particle model is only 4×10^{-2} explosions $\text{pc}^{-3} \text{ yr}^{-1}$ (ref. 3), it seems likely that Cerenkov, particle, or satellite γ -ray detectors will probably not produce limits lower than about 10^3 explosions $\text{pc}^{-3} \text{ yr}^{-1}$ when the elementary particle model is assumed.

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A non-singular quantum Universe

CLASSICAL relativistic singularities remain one of the most puzzling problems of contemporary physics. Although no consensus of opinion has been attained on their status, a great deal of work has been devoted to the search for physical mechanisms capable of eliminating the cosmological singularities, whose existence follows from the theorems of Hawking and Penrose^{1,2}. The alternative view that the initial singularity of big-bang models is essential for the coming into being of the Universe, has also been advocated^{3,4}. Some suggestions of cosmological singularity avoidance have recently come to light, in the 'classical' quantum regime (from 10^{-43} – 10^{-23} s) (refs 5–8) or even without resorting to quantum effects⁹. The quantum gravitational period ($t < 10^{-43}$ s) remains potentially very interesting for searching for singularity avoidance mechanisms. An interesting approach is that of quantum cosmology, based on the hamiltonian formulation of general relativity (Dirac's¹⁰ or Arnowitt, Deser and Misner's¹¹ (ADM) method). This approach was initiated by De Witt¹² and then followed by Misner¹³ and many others (see refs 14,15) and is used here to derive a non-singular quantum cosmological model.

Much of the work done in quantum cosmology has been concerned with empty models. De Witt¹², however, in his particles treatment of a closed Friedmann Robertson-Walker (FRW) Universe, introduced a cloud of dust particles to support this metric. The initial singularity is avoided artificially in this model by postulating the existence of an infinitely steep wall at $R = 0$, (R is the 'radius' of FRW Universe) to exclude negative values of R . This choice forces the quantum wave function of the Universe to be zero at $R = 0$.

Misner's¹³ pioneering work about quantum vacuum homogeneous models (Bianchi I and IX Universes), based on the ADM formalism, shows that the quantum gravitational fluctuations offer these models no escape from the classical singularity. The same conclusion seems to be true for the Bianchi Universes filled with a perfect gas with an equation of state of the type $p = (\gamma - 1)\rho$ (p and ρ denote respectively the pressure and total density of matter and γ is a constant between 1 and 2) if the matter is described by Ryan's¹⁴ lagrangian which introduces no additional dynamical degree of freedom for the perfect gas. (Units are chosen so that $c = 1$, $G = 1$ and $\hbar/2\pi = 1$.)

On the other hand, Schutz^{16,17} has built a hamiltonian theory of the relativistic perfect fluid, based on the velocity-potential version of perfect-fluid hydrodynamics of Seliger and Whitham¹⁸. Schutz's canonical formalism introduces as dynamical degrees of freedom for the fluid the velocity potentials.

Lund¹⁹ has used Schutz's formalism to study the effects of quantum gravitational fluctuations on the final issue of collapse of balls of dust ($p = 0$) and on the singularity of a closed dust-filled FRW Universe. He has taken the time variable from the matter variables, while Ryan's¹² time coordinate was Misner's¹³ gravitational degree of freedom Ω . Ryan's method leads to a Klein-Gordon type equation for the wave function of the Universe, with its well known difficulties of interpretation while Lund's choice of time leads, in the cases he has considered, to a Schrödinger-like equation amenable to a probability interpretation (see ref. 20). By imposing the requirement that the corresponding mini-superspace²⁰⁻²² be geodesically complete, Lund concludes that, in this formalism, there is no inevitable singularity.

However, this Schrödinger equation, in the case of the closed FRW model, cannot be analytically solved and there is no guarantee that the wave function becomes zero for $R = 0$, which would be the case if it were impossible to find the Universe in the singular state. (For another possible definition of the absence of singularity in a quantum cosmological model, see ref. 23).

Thus, as far as we know, there has been no convincing evidence in the framework of quantum cosmology that the classical singularity could be avoided, under the influence of quantum gravitational fluctuations²⁴.

We give here an example of a non-singular quantum cosmological model, the 'dust-filled' Bianchi I Universe, "a behavioural paradigm for all anisotropic cosmological models"²⁵, quantised following the ADM method and with Lund's choice of time coordinate. The advantage of this model is that it can be analytically solved. As regards the hypothesis of a null pressure, it corresponds to the soft equation of state resulting from the Hagedorn²⁶ composite particle picture and it can also represent a cold, low-entropy early Universe filled with non-interacting baryons^{27,28}.

We use for the flat three-space theory of the Bianchi I Universe, Misner's¹³ form:

$$dl^2 = R_0^2 e^{2\mu} \{ \exp[2(\beta_+ + 3^{1/2}\beta_-)](dx^1)^2 + \exp[2(\beta_+ - 3^{1/2}\beta_-)](dx^2)^2 + \exp[-4\beta_+](dx^3)^2 \} \quad (1)$$

R_0 is a constant and μ , β_+ and β_- are functions only of a time coordinate to be determined; μ , β_+ and β_- will be considered as the dynamical degrees of freedom.

We choose for the canonically conjugate moments to the g_{ij} , that is π^{ij} , the following expressions

$$\pi^{11} = \left\{ \frac{1}{12} [\pi_+ + 3^{1/2}\pi_-] + \frac{\pi_\mu}{6} \right\} \times R_0^{-2} e^{-2\mu} \exp[-2(\beta_+ + 3^{1/2}\beta_-)] \quad (2a)$$

$$\pi^{22} = \left\{ \frac{1}{12} [\pi_+ - 3^{1/2}\pi_-] + \frac{\pi_\mu}{6} \right\} \times R_0^{-2} e^{-2\mu} \exp[-2(\beta_+ - 3^{1/2}\beta_-)] \quad (2b)$$

$$\pi^{33} = \left\{ -\frac{\pi_+}{6} + \frac{\pi_\mu}{6} \right\} R_0^{-2} e^{-2\mu} e^{4\beta_+} \quad (2c)$$

where π_μ , π_+ and π_- are the momenta conjugate to μ , β_+ and β_- , respectively. The action for the gravitational field and the matter (dust) can then be written as

$$S = \pi \int [\pi_\mu \dot{\mu} + \pi_+ \dot{\beta}_+ + \pi_- \dot{\beta}_- + p^\phi \dot{\phi} - N(\mathcal{H}_V^0 + \mathcal{H}_M^0) - N_i(\mathcal{H}_V^i + \mathcal{H}_M^i)] dt \quad (3)$$

$\mathcal{H}_V^0$ and $\mathcal{H}_M^0$ are respectively the super-hamiltonian constraints for the vacuum¹⁹ and for dust^{16,19}

$$\mathcal{H}_V^0 = \frac{R_0^{-3} e^{-3\mu}}{24} [\pi_+^2 + \pi_-^2 - \pi_\mu^2] \quad (4a)$$

$$\mathcal{H}_M^0 = 16\pi g^{1/2} N^2 (u^0)^2 \rho_0 = -N(u^0)p^\phi \quad (4b)$$

where ϕ is the only Seliger-Whitham velocity potential different from zero in this case; it is related to the four-velocity vector u_ν by

$$u_\nu = h^{-1} \frac{\partial \phi}{\partial x^\nu} \quad (5)$$

where h is the specific enthalpy of the fluid:

$$h = \frac{\rho + p}{\rho_0} \quad (6)$$

ρ_0 denoting the rest-mass density; p^ϕ is the momentum canonically conjugate to ϕ and N and N_i , respectively, the ADM lapse and shift functions; g denotes the determinant of the covariant spatial metric tensor and the overdots, in equation (3), the derivation with respect to the time variable, t .

Due to the homogeneity property of the $t = \text{constant}$ surfaces of Bianchi-type universes, the supermomentum constraints: $\mathcal{H}_V^i + \mathcal{H}_M^i = 0$ are automatically satisfied.

Lund's method consists in choosing as time variable, the velocity potential ϕ :

$$T(t) = -\phi(t) \quad (7)$$

and imposing, in the framework of the ADM method, the coordinate condition:

$$t = T = -\phi \quad (8)$$

Then, solving the constraint: $\mathcal{H}_V^0 + \mathcal{H}_M^0 = 0$ for p^ϕ , we get for equation (3)

$$S = \pi \int [\pi_\mu \dot{\mu} + \pi_+ \dot{\beta}_+ + \pi_- \dot{\beta}_- - H_{\text{ADM}}] dt \quad (9)$$

where the ADM hamiltonian, H_{ADM} is given by

$$H_{\text{ADM}} = p^\phi = \frac{R_0^{-3} e^{-3\mu}}{24} [\pi_+^2 + \pi_-^2 - \pi_\mu^2] \quad (10)$$

To quantise this model, we have to be careful: the usual choice of operators¹⁴ corresponding to the classical conjugate momenta, for instance $\hat{\pi}_\mu = (1/i)(\partial/\partial\mu)$ is not appropriate here, as it would lead to a non-self-adjoint hamiltonian operator. However, if we introduce the canonical transformation

$$x = 4\left(\frac{2}{3}\right)^{1/2} e^{3\mu/2} \\ \pi_x = \frac{1}{4}\left(\frac{2}{3}\right)^{1/2} e^{3\mu/2} \pi_\mu \quad (11)$$

the following hamiltonian operator

$$\hat{H}_{\text{ADM}} = R_0^{-3} \frac{\partial^2}{\partial x^2} - \frac{4R_0^{-3}}{9x^2} \left[\frac{\partial^2}{\partial \beta_+^2} + \frac{\partial^2}{\partial \beta_-^2} \right] \quad (12)$$

is self-adjoint with respect to the following inner product of wave functions of the Universe

$$\langle \Phi | \Psi \rangle = \int \int \int \Phi^*(x, \beta_+, \beta_-) \Psi(x, \beta_+, \beta_-) dx d\beta_+ d\beta_- \quad (13)$$

The Schrödinger equation to be solved for the wave function of the Bianchi I Universe is then

$$i \frac{\partial \Psi}{\partial t} = R_0^{-3} \left[\frac{\partial^2 \Psi}{\partial x^2} - \frac{4}{9x^2} \left(\frac{\partial^2 \Psi}{\partial \beta_+^2} + \frac{\partial^2 \Psi}{\partial \beta_-^2} \right) \right] \quad (14)$$

whose acceptable solutions for Ψ must be bounded in the complete domain of definition of the variable x, β_+ and β_- , that is

$$x \in [0, +\infty], \beta_+ \in [-\infty, +\infty] \text{ and } \beta_- \in [-\infty, +\infty].$$

The general solution Ψ of equation (14) can be written as a superimposition of particular solutions for the values E, p_+ and p_- of the separation constants

$$\begin{aligned} \Psi(x, \beta_+, \beta_-, t) &= \alpha \int \int \int C(E, p_+, p_-) \Psi_{E, p_+, p_-}(x, \beta_+, \beta_-, t) \\ &\quad dE dp_+ dp_- = \alpha \int \int \int C(E, p_+, p_-) e^{-iEt} \cdot \exp[i(p_+ \beta_+ + p_- \beta_-)] \\ &\quad \cdot Y_{E, p_+, p_-}(x) dE dp_+ dp_- \end{aligned} \quad (15)$$

where $C(E, p_+, p_-)$ is the amplitude of the eigenstate of particular values $E (E < 0$; see equations (4b) and (10) see also Moncrief³⁰), p_+ and p_- of the separation constants; α is an overall normalisation constant and $Y_{E, p_+, p_-}(x)$ is solution of

$$x^2 \frac{d^2 Y}{dx^2} + [\frac{4}{3}(p_+^2 + p_-^2) + R_0^3 |E| x^2] Y = 0 \quad (16)$$

This solution can be written as follows

$$\begin{aligned} Y_{E, p_+, p_-} &= \bar{\alpha} x^{1/2} [AJ + (\frac{4}{3} - \frac{4}{3}(p_+^2 + p_-^2))^{1/2} (xC^{1/2}) \\ &\quad + BJ - (\frac{4}{3} - \frac{4}{3}(p_+^2 + p_-^2))^{1/2} (xC^{1/2})] \end{aligned} \quad (17a)$$

With

$$C = |E| R_0^3 \quad (17b)$$

Where J_ν is the Bessel function of order ν . A and B are constants determined by initial conditions and $\bar{\alpha}$ a constant of normalisation such that

$$\langle \Psi_{E, p_+, p_-} | \Psi_{E', p'_+, p'_-} \rangle = \delta(E - E') \delta(p_+ - p'_+) \delta(p_- - p'_-)$$

This solution is acceptable due to the asymptotic behaviour of $J_\nu(z)$ for $|z| \rightarrow \infty$. Moreover, by taking into account the behaviour of $J_\nu(z)$ for $z \rightarrow 0$, one deduces that

$$\Psi_{E, p_+, p_-}^* \Psi_{E, p_+, p_-} \xrightarrow{x \rightarrow 0} 0 \quad (18)$$

We conclude that for a 'dust-filled' Bianchi I cosmological model, the classical singularity is avoided due to the presence of quantum gravitational effects in the sense that because $\Psi^* \Psi(x = 0) = 0$, it is impossible to find this Universe in the singular state.

For the isotropic version of the 'dust-filled' Bianchi I model, that is for the 'dust-filled' Einstein-de Sitter model ($\beta_+ = \beta_- = 0$), the wave function becomes zero at $x = 0$ only for a set of measure zero of models, which means that, in this case, the singularity will not be avoided.

The cause of possible singularity avoidance in the 'classical' quantum (or classical) regime⁵⁻⁹, is usually ascribed to a violation of the energy condition of Hawking and Penrose's^{1,2} theorems. We feel, however, that the non-singular character of the Bianchi I model studied here in the framework of quantum

cosmology may be due to the fact that the quantum gravitational fluctuations do not allow the model to be sufficiently localised. Moreover, the singular Einstein-de Sitter models may be considered as very improbable (structurally unstable) models in the ensemble of all anisotropic Bianchi I Universes.

It would be interesting to extend the present work to the case of Bianchi-type models filled with a perfect fluid of equation of state: $p = (\gamma - 1)\rho$, which covers Zel'dovich's³⁰ stiff equation of state ($p = \rho$) as well as the hard equation ($p = \frac{1}{3}\rho$) characteristic of Collins and Perry's³¹ 'quark soup'. This analysis would perhaps allow us to conclude as regards the truth of Wheeler's³² conjecture "that every quantum solution for the problem of a closed universe within the framework of Einstein's field equations leads to a singularity". Work in this direction using Moncrief's²⁹ recent hamiltonian formalism for perfect fluids and Schutz's¹⁷ formalism is in progress.

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Stability of quadruple junctions

THE analysis of triple junctions (points on the Earth where three lithospheric plates meet) has proved to be a powerful tool in deciphering the plate tectonic evolution of the Earth. In their classic paper on the evolution of triple junctions¹, McKenzie and Morgan dismissed the possibility of stable quadruple junctions existing more than instantaneously. We show here that quadruple junctions can be stable according to the vector velocity stability criteria of McKenzie and Morgan.

Consider the quadruple junction configuration of Fig. 1. Both spreading centres (A-B and C-D) extending into the junction

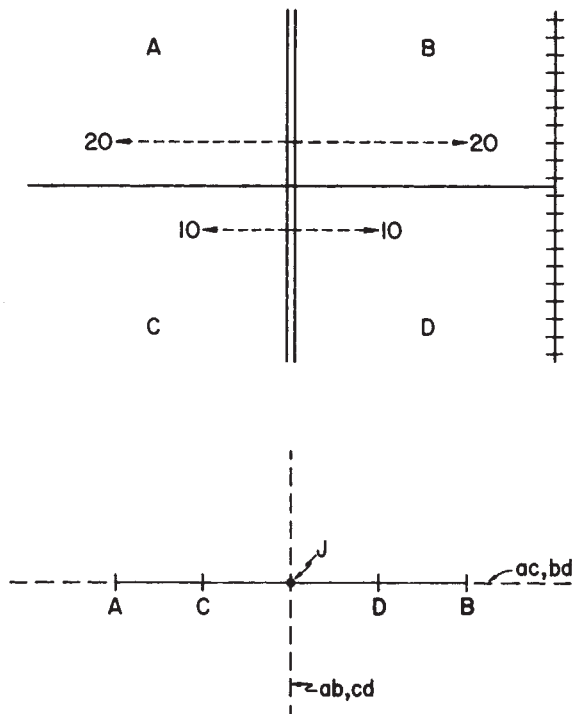


Fig. 1 Example of a stable quadruple junction and its representation in velocity space. Double lines are ridges, single lines are transform faults, hatchured line is trench. Arrows show relative plate motions. J represents quadruple junction.

spread symmetrically at the rates shown. Plate A therefore slips 10 mm yr^{-1} west relative to plate C and plate B slips 10 mm yr^{-1} east relative to plate D. This RFRF junction can clearly evolve continuously in this geometric configuration, which is confirmed by vector velocity analysis¹ (Fig. 1) or with scissors and paper. It is thus stable in the McKenzie-Morgan sense.

Many other stable quadruple junction configurations have been calculated by one of us (P.M.). Only the TFFF and FFFF junctions have no possible stable configuration. We note that stable quadruple and higher order n -tuple ridge junctions are possible if all n plate velocities fall along a circle in velocity space. All relative plate motions would be represented by chords of the circle, and the junction would have the vector velocity of the centre of the circle. Note that if this velocity criterion is met, the ridges can be at any orientations and the junction will be stable (the total angular spread of the ridges must be more than 180° if spreading is required to be non-oblique on all ridges; see ref. 3). If all four plate boundaries are orthogonal, five possible stable quadruple junctions may exist which do not require a specific ratio of plate velocities (RRRR, RFRF, RTRT, TTTF, and TTTT). In each of these cases, however, plate velocities are somewhat constrained by the geometric requirements; spreading is required to be symmetric and non-oblique, all transform faults are pure strike-slip, some subduction is required to be oblique and some non-oblique, and the subducted plate at each trench must be specified. These junctions are the only quadruple junctions which do not require both a specific geometry and specific plate velocities for stability. A possible situation in which a RRRR junction might be formed is during initial doming and rifting of a continent. If this happened, the subsequent establishment of normal seafloor spreading could result in a pair of failed rifts (aulocogenes) extending away from the quadruple junction.

There is a near quadruple junction today where the Pacific, Philippine, China and Australian plates nearly come together at the southern tip of the Philippine plate. There is also a near-juncture of the Pacific, Cocos, Rivera, and North America plates near the mouth of the Gulf of California. This could be a stable

quadruple junction fixed with respect to the Rivera plate if the Pacific-Cocos spreading centre had the same vector velocity as the Rivera plate.

We agree with McKenzie and Morgan that quadruple junctions are unlikely to be of much importance in the Earth's evolutionary history, but a necessary step in identifying possible quadruple junctions is the recognition that they may exist.

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Charge transfer by surface states in the photoelectrolysis of water using a semiconductor electrode

THE photoelectrolysis of water using n -type semiconductors such as TiO_2 , (refs 1-4) SrTiO_3 , (refs 5-7) and so on is of current interest and importance for the utilisation of solar energy to produce hydrogen gas for fuel. In steady state conditions under illumination and in the absence of H_2 and O_2 in the electrolyte, the rate of H_2 evolution on a cathode is proportional to that of O_2 evolution on a semiconductor anode. The O_2 evolution process is considered to proceed by electron tunnelling from electrolyte levels to holes in the semiconductor through the Helmholtz layer, which must take place between states of equal energy. If only isoenergetic electron transfer between holes and occupied electrolyte states overlapping in energy the valence band occurs, the hole currents for O_2 evolution at $n\text{-TiO}_2$ would be expected to be very low, because the equilibrium redox level of the $\text{H}_2\text{O}/\text{O}_2$ couple is considerably above (at 1.8 eV for $\text{pH} = 4.7$ (ref. 2)) the top of the valence band. Experimentally, however, the current of holes at the TiO_2 -electrolyte interface is found to be quite high and the efficiency in charge transfer of holes at the interface is almost 100%⁸. To account for such a high efficiency, a contribution from some surface states has been proposed^{8,9}. A possible importance of surface states in electrochemistry at TiO_2 electrodes has been described by Frank and Bard¹⁰. Also, a recent electroluminescence measurement made for $n\text{-TiO}_2$ indicates that occupied surface states exist near the midgap at the TiO_2 -electrolyte interface¹¹. At present, however, it is not understood which kind or character of surface states can improve the charge transfer efficiency for holes at the interface. We present here a theoretical treatment of electron tunnelling between the semiconductor and electrolyte by surface states. We apply a theory of surface states (more exactly interface states) in tunnel MOS (metal-oxide-semiconductor) devices of Freeman and Dahlke¹² to the semiconductor-electrolyte interface. A quite high efficiency for holes at the interface can be obtained when the electron population of the surface states is controlled by the electrolyte and the surface states are almost completely occupied.

The energy band diagram of an n -type semiconductor in an electrolyte under hole injection (by illumination) is shown in Fig. 1. For simplicity we assume that the surface states can be described by a single energy level E_s with a surface density of N_s . The charging of the surface states can take place by interaction with electrons in the conduction and valence bands of the semiconductor and by tunnelling of electrons between the surface states and electrolyte. In the steady state condition, the total

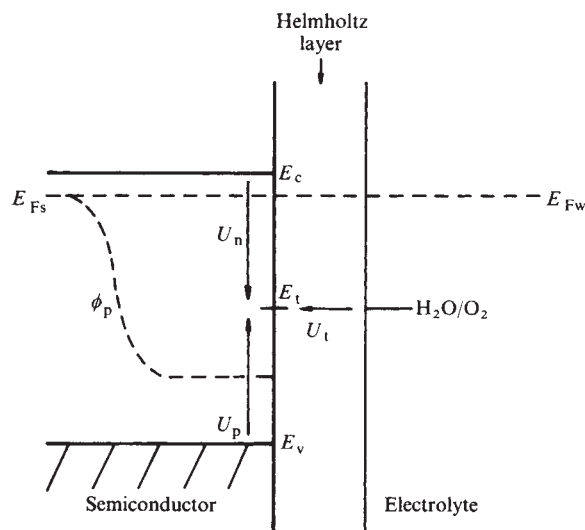


Fig. 1 Energy-level diagram for an n-type semiconductor-electrolyte interface with surface states at energy E_t and $\text{H}_2\text{O}/\text{O}_2$ redox level. The level E_{Fw} in the electrolyte and the Fermi level for electrons E_{Fs} in the semiconductor are aligned. The change in the quasi-Fermi level for holes ϕ_p is greatly exaggerated for clarity and indicates an increase in concentration of holes in the surface region of the semiconductor under illumination.

flux of electrons into the surface states must be equal zero. Then, the electron tunnel flux from the electrolyte into the surface states through the Helmholtz layer, which is proportional to the O_2 evolution rate, can be described as¹²

$$U_t = N_t(f_w - f_0)/(\tau_t + \tau_0) \quad (1)$$

Here, τ_t is a tunnelling time constant, f_w is an occupancy of the electrolyte states, and τ_0 and f_0 are constants characteristic of the surface recombination without tunnelling and are given by, respectively,

$$\tau_0 = [C_p(p_s + p_1) + C_n(n_s + n_1)]^{-1} \quad (2)$$

and

$$f_0 = \tau_0(C_p p_1 + C_n n_s) \quad (3)$$

where C_n and C_p are electron and hole capture coefficients, n_s and p_s are electron and hole concentrations at the surface of the semiconductor, and n_1 and p_1 are concentrations of electrons and holes to be expected when the Fermi level is at the surface state level.

If we assume that $\tau_t \ll \tau_0$ and $f_w = 1$, we have from equation (1)

$$U_t = N_t(C_p p_s + C_n n_1) \quad (4)$$

These conditions result in the situation that the electron population of the surface states is controlled by the electrolyte and the surface states are almost completely occupied. Furthermore, the condition of $\tau_t \ll \tau_0$ implies that the capture coefficients C_n and C_p in equation (2) should be sufficiently small.

Equation (4) indicates that U_t is determined by the hole capture from the valence band to the surface states and the electron emission from the surface states to the conduction band. In other words, an electron which tunnels from the electrolyte into the surface states either recombines with a hole in the valence band or enters the conduction band. The latter may be neglected compared with the former under illumination by which the surface concentration of holes p_s is increased, as n_1 is small when the surface state level is located near the midgap. Thus, U_t , that is the O_2 evolution rate is determined by the rate at which the almost completely occupied surface states capture holes at the semiconductor surface and is proportional to the surface concentration of holes, being in turn proportional to the

light intensity of illumination, as observed experimentally. Because the hole emission from the surface states to the valence band is neglected in these conditions, it follows that all the holes reaching the semiconductor surface can be captured by the surface states and recombine with electrons in the electrolyte with the transfer probability of one. This can result in quite high efficiencies in charge transfer of holes at the interface.

Both the conditions of $\tau_t \ll \tau_0$ and $f_w = 1$ can be satisfied by the n- TiO_2 electrode, as the Helmholtz layer existing on the electrode is thin enough (of the order of 1 Å) to satisfy $\tau_t \ll \tau_0$ although the capture coefficients in TiO_2 are unknown, and $E_t = 1.5 \sim 1.24$ eV (ref. 11) and $E_{Fw} = 2.3$ eV (ref. 2) above the top of the valence band gives $f_w = 1$, where E_{Fw} corresponds to the Fermi level in the electrolyte. This explains the quite high transfer efficiencies observed at the TiO_2 -electrolyte interface. But, it is not clear whether the surface states closely related to charge transfer at the TiO_2 -electrolyte interface are pre-existing ones associated with surface $\text{Ti}^{3+}(3d^1)$ species^{13,14} or some localised states occurring as a result of chemical bonding between the electrode and electrolyte. To clarify this point, further experiments for charge transfer at the interface using TiO_2 or SrTiO_3 electrodes with and without pre-existing surface states would be desired.

Finally, if we assume $\tau_t \gg \tau_0$, the U_t value is only a small fraction τ_0/τ_t of that for the case of $\tau_t \ll \tau_0$ and very low efficiencies in charge transfer are predicted.

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High frequency radar observations of horizontal plasma waves in the equatorial ionosphere

THE anomaly observed on the magnetic field records in equatorial zones is due to a strong ionospheric current flowing around 105 km, called the equatorial electrojet. We have used coherent radar returns from electron density irregularities embedded in this current to measure electric fields in this region and to study plasma instabilities at the origin of these irregularities. We report here that strong anomalous reversals of the equatorial electrojet are sometimes observed and quasi-horizontal plasma waves have been detected for the first time during such an event.

Plasma instabilities have been previously investigated in the equatorial electrojet using VHF and HF radars^{1,2} and *in situ* measurements during rocket launches³. While a good height resolution is obtained with rocket measurements, wave spectrum information has only been obtained through radar measurements. However, due to Earth curvature, the classical VHF radar, as used at 50 MHz in South America, is not adapted to detect plasma waves moving at grazing angles below about

10° at the electrojet altitude of 105 km (ref. 4). It is only when HF radars are used that the refraction in the E region of the lower frequency radio rays is sufficient for a possible detection of these horizontal plasma waves, the properties of which are known from the theory to be specially interesting⁴.

Such an HF experiment has been developed near the magnetic equator at Addis Ababa (Ethiopia) since 1976 using a phase coherent HF radar operating at 7, 14, 21 and 29 MHz. At the lower frequencies, radio rays are usually totally reflected by the E layer during daytime but, while classical oblique radar echoes have been observed almost every day² and identified as the so-called 'type 1' or 'type 2' echoes discovered previously at VHF¹, nothing typical was ever detected related to horizontal radio rays. It is only during a very strong counter-electrojet event described elsewhere⁵ that very anomalous echoes were found to be associated with the horizontal radio rays.

On 21 January 1977 coherent HF radar measurements were performed for the first time during a strong counter-electrojet. During such an event, the upward electron density gradient of the E layer is no longer destabilising due to the reversal of the electric field⁶ and the cross-field instability cannot grow; however, the electron velocity is large enough (larger than the ion acoustic velocity in the medium) for the two stream instability to grow. It was then the first opportunity to investigate this instability without a background of turbulence due to the cross-field instability.

The spectral analysis of the radar echoes obtained at 14 MHz was performed at different time delays after the transmitted pulse corresponding to a variation of the zenith angle and a selection of different instability wave vectors (Fig. 1). The most striking and unexpected result is the discontinuity in both phase velocity and cross-section as a function of range and the simultaneous occurrence of two echoes for some ranges.

At the shortest ranges (or more oblique wave vectors) the phase velocity is larger and the cross-section much smaller than at the largest ranges (or more horizontal wave vectors). The shortest range echoes are similar to the usual 'type 1' echoes and are labelled accordingly (Fig. 1).

These experimental results can be considered in the light of a ray tracing of radio wave propagation for the conditions prevailing in the E layer at the time of the observations ($f_{oE} = 3$ MHz, $H_{max} = 105$ km). It is found (Fig. 2) that radio rays can be horizontal further than about 600 km, while strong refraction is still observed between 450 and 600 km. The strong amplitude and low phase velocity echoes labelled 'type H' on Fig. 1 can

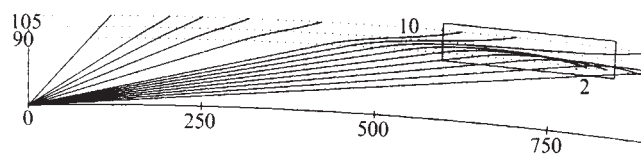


Fig. 2 Radio ray tracing at 14 MHz using a parabolic E layer ($f_{oE} = 3$ MHz, $h_{max} = 105$ km, $H = 7.5$ km) showing the horizontal radio rays further than 500 km.

then be associated with horizontal and quasi-horizontal plasma wave vectors with a maximum cross-section for the horizontal waves. The association of the 'type H' echoes with the horizontal waves is confirmed by the time variation of the location of the amplitude maximum: the range of this maximum increases with time as given by the ray tracing when the E layer critical frequency decreases (that is, totally refracted echoes are observed at greater ranges).

The 'type H' echoes observed during the 21 January counter-electrojet event can then be associated with horizontal or quasi-horizontal plasma waves. The fact that such a phenomenon is not observed during normal electrojet conditions could be due to the turbulent state of the current flow when long wavelength cross-field instabilities grow in the normal daytime electrojet. When the electric field and the current are reversed during the counter-electrojet with no modification of the upward electron density gradient, the cross-field instability cannot grow and the turbulent mixing does not appear. The current flow is then always in a laminar state and the first waves appearing (the horizontal ones) will not have their growth rate modified by the turbulence as during normal electrojet conditions.

The characteristics of the 'type H' echoes cannot be definitely associated with either two stream (type 1) or cross-field (type 2) instabilities. On the one hand, the very narrow width of their spectrum, the strong amplitude and the lack of correlation between the phase velocity and the magnetic field variation are typical of type 1 spectra; on the other hand, the relatively low value of the phase velocity and the presence at the bottom of the layer are typical of type 2 echoes.

Among the two striking characteristics of type H echoes, the very strong amplitude is certainly associated with the fact that in a laminar electrojet the horizontal waves have the largest growth rate, but the other characteristic, the low value of the phase velocity, is still very puzzling and may be related either to some saturation effect similar to the quasi-linear reduction of the electric field⁴ or to some secondary phenomena in the electrojet known to modify the phase velocity of the instabilities such as the presence of a strong neutral wind gradient with altitude or longitude or the occurrence of an anomalous gradient related to a thin blanketing sporadic E⁷.

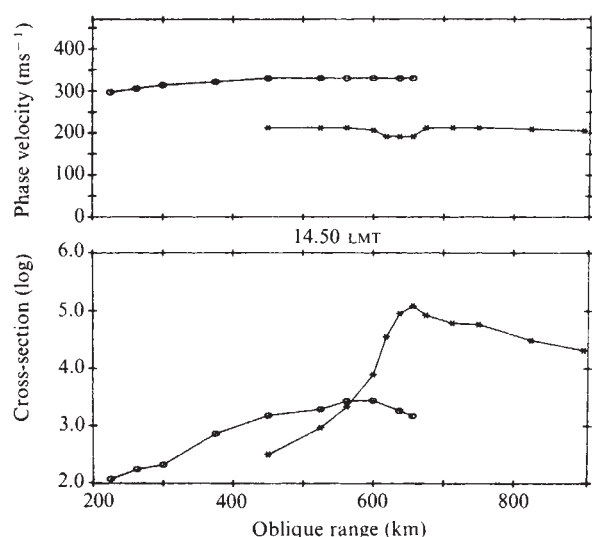
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Fig. 1 Phase velocity and scattering cross-section variation for type 1(○) and type H (*) echoes with the oblique range (in km) in the eastward direction at 14.50 LT on 21 January 1977 at Addis Ababa.



Gradual increase of oceanic CO₂

SINCE 1850, human activities, mainly the burning of fossil fuels and the clearing of forests, have increased the amount of CO₂ in the atmosphere by ~50 p.p.m.¹⁻⁵. Although it is generally believed that the oceanic CO₂ has increased correspondingly⁶⁻¹¹, only very limited data¹²⁻¹⁴ have shown the increase quantitatively. We present here a computational calculation of the variation of the preformed total CO₂ (ΣCO_2^0) for a water mass, the original amount of total CO₂ (ΣCO_2) at the time of its last contact with the atmosphere. The result for the Atlantic Ocean reveals that the older water masses have invariably smaller ΣCO_2^0 as compared to the more recently formed waters.

Assuming a parcel of seawater in contact with the air remains at a constant degree of equilibrium¹⁵ with the atmospheric CO₂, the amount of ΣCO_2 in this water is defined as ΣCO_2^0 , which depends on the partial CO₂ pressure in the air (p_{CO_2}) as well as the salinity and the temperature of the seawater. Once the water sinks, the *in situ* decomposition of particulate CaCO₃ and organic carbon adds dissolved CO₂ to the parcel^{16,17}. Consequently, the measured ΣCO_2 becomes larger than ΣCO_2^0 . The values of ΣCO_2^0 can only be calculated from the measured ΣCO_2 values by correcting for the influences of CaCO₃ and organic carbon, which in turn can be calculated using the titration alkalinity (TA) and the apparent oxygen utilisation (AOU) data. (The combined effect of decomposing x moles of CaCO₃ and y moles of organic matter in 1 kg of seawater on ΣCO_2^0 , TA⁰ and AOU can be represented as^{16,17}:

$$\Sigma\text{CO}_2 - \Sigma\text{CO}_2^0 = x + 106y$$

$$\text{TA} - \text{TA}^0 = 2x - 17y$$

$$\text{AOU} = 138y$$

Eliminating x and y from the above equations yields equation (1). Note that the constant associated with AOU is 0.83 instead of the often misused 0.768.)

$$\Sigma\text{CO}_2^0 = \Sigma\text{CO}_2 (\text{measured}) - 0.5[\text{TA}(\text{measured}) - \text{TA}^0] - 0.83 \text{ AOU} \quad (1)$$

where TA⁰ is the preformed TA value. The values for TA⁰ probably do not vary with the atmospheric CO₂ variation but depend only on the salinity and temperature. Therefore, we have assumed that the present day surface TA values represent the values for TA⁰ during the past 130 yr.

The Geosecs (Geochemical Ocean Section Study) Atlantic surface TA data have been fitted to a function of potential temperature (θ)¹⁸.

We have calculated TA⁰ (and other chemical properties) on the constant salinity (35‰) basis. The values of chemical properties, for example TA⁰, are normalised to 35‰ salinity (S) using:

$$\text{TA}^0 = \frac{\text{TA}^0(\text{measured}) \times S(\%, \text{measured})}{35.000}$$

$$\begin{aligned} \text{TA}^0(\mu\text{Equiv. kg}^{-1}) &= 2,368 - 4.72\theta, \quad \theta < 17^\circ\text{C} \\ &= 2,287, \quad \theta > 17^\circ\text{C} \end{aligned} \quad (2)$$

Combining equations (1) and (2) gives:

$$\begin{aligned} \Sigma\text{CO}_2^0(\mu\text{mol kg}^{-1}) &= \Sigma\text{CO}_2(\text{measured}) - 0.5\text{TA}(\text{measured}) \\ &\quad - 0.83 \text{ AOU} + 1,184 - 2.36\theta, \quad \theta < 17^\circ\text{C} \\ &= \Sigma\text{CO}_2(\text{measured}) - 0.5\text{TA}(\text{measured}) \\ &\quad - 0.83 \text{ AOU} + 1,143.5, \quad \theta > 17^\circ\text{C} \end{aligned} \quad (3)$$

The values for ΣCO_2^0 for the Atlantic waters formed in 1972

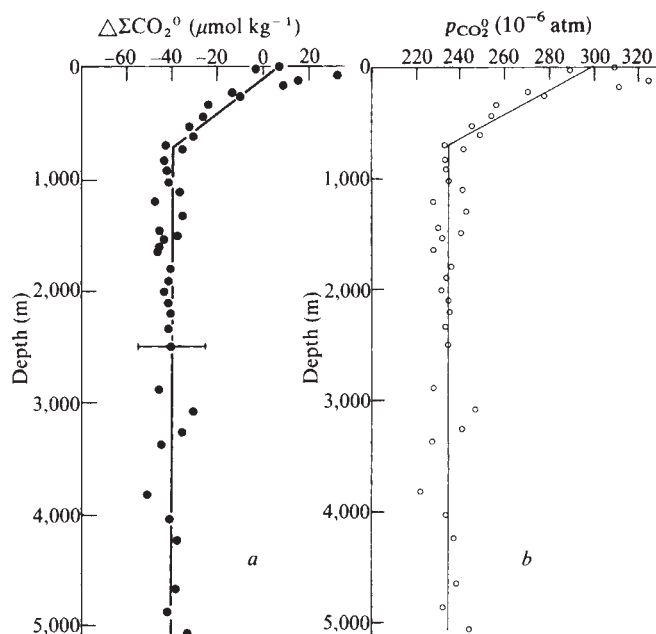


Fig. 1 Variations of $\Delta\Sigma\text{CO}_2^0$ (a) and p_{CO_2} (b) with depth at 59°56.5' S, 45°2.0' W.

have been assumed to be equal to the surface ΣCO_2 values determined in that year (Geosecs). They are represented by

$$\Sigma\text{CO}_2^0(\mu\text{mol kg}^{-1}, 1972) = 2,195 - 9.5\theta \quad (4)$$

The difference in ΣCO_2^0 ($\Delta\Sigma\text{CO}_2^0$) between the older water masses calculated from equation (3) and the present day (that is 1972) values calculated from equation (4) is probably caused by changes in the atmospheric CO₂ concentrations. The uncertainty in $\Delta\Sigma\text{CO}_2^0$ is estimated to be $\pm 15 \mu\text{mol kg}^{-1}$ due to the uncertainties in the measured chemical properties, namely ΣCO_2 , TA and AOU.

The vertical variation of $\Delta\Sigma\text{CO}_2^0$ was calculated from a station in the Southern Ocean (Geosecs station 79; 59°56.5' S, 45°2.0' W). The values for $\Delta\Sigma\text{CO}_2^0$ decrease from approximately zero to nearly $-40 \pm 15 \mu\text{mol kg}^{-1}$ from the surface to 700 m, but remain constant from 700 m to 4,860 m (Fig. 1a). The decrease of roughly $40 \pm 15 \mu\text{mol kg}^{-1}$ in $\Delta\Sigma\text{CO}_2^0$ with depth for the relatively young shallow waters reflects the decrease of atmospheric CO₂ with age. The deeper layer does not show depth-dependent variations in $\Delta\Sigma\text{CO}_2^0$, presumably because it was formed before the atmospheric CO₂ started to increase. The vertical profile for p_{CO_2} (Fig. 1b) looks similar to the profile for $\Delta\Sigma\text{CO}_2^0$ and shows a decrease of 65 ± 30 p.p.m. from the surface to 700 m. Very little tritium, but large negative values for $\Delta^{14}\text{C}$ were found^{19,20} below 600 m for waters south of 55° S, which confirms our conclusion. The variation in p_{CO_2} is larger than the value generally accepted¹⁻⁵ or the value (50 p.p.m.) obtained by Brewer¹⁴ using a similar approach.

Similar horizontal propagations of $\Delta\Sigma\text{CO}_2^0$ with latitude have been traced along the minimum salinity (Fig. 2a) and the minimum potential temperature (Fig. 2b) layers in the Atlantic Ocean. Figure 2a shows that the values for $\Delta\Sigma\text{CO}_2^0$ generally decrease from 45° to 20° S in the minimum salinity layer (~800 m deep). The break near 38° S is probably due to the change in flow patterns^{21,22}, as northwards flowing minimum salinity water turns eastwards at about 40° S and returns around the anticyclonic gyre. The water north of 38° S is significantly older than the water south of 38° S. There is probably another break at 22° S. The values of $\Delta\Sigma\text{CO}_2^0$ for water between 22° S and 25° N remain relatively constant at $-40 \pm 15 \mu\text{mol kg}^{-1}$, indicating that there was no significant CO₂ variation in the atmosphere when this parcel of water was formed. The values

for $\Delta\Sigma\text{CO}_2^0$ increase with latitude north of 25°N due to the influence of the freshly formed North Atlantic Deep Water²³. There are no significant differences between the waters in the western and eastern Atlantic Ocean.

The tritium data support our findings. The average tritium value is 0.47 tritium unit (TU) at the depths of interest south of 38°S . This value is higher than the average value of 0.15 found between 38° and 22°S , which in turn, is higher than the average value of 0.06 TU for stations between 22°S and 25°N . Tritium concentrations are high for stations north of 25°N .

The values for $\Delta\Sigma\text{CO}_2^0$ in the minimum potential temperature layer (roughly 4,500 m deep) are shown in Fig. 2b. Waters in the eastern basin all have $\Delta\Sigma\text{CO}_2^0$ values of approximately $-40 \pm 15 \mu\text{mol kg}^{-1}$, indicating that these waters formed before the industrialisation. Waters in the western basin are apparently younger. The value of $\Delta\Sigma\text{CO}_2^0$ is $\sim -20 \pm 15 \mu\text{mol kg}^{-1}$ near 50°S in the western basin which suggests that the water here has been influenced by the atmospheric CO_2 increase. In the south, values for $\Delta\Sigma\text{CO}_2^0$ show a northwards decrease in the western basin. The values decrease to $-40 \pm 15 \mu\text{mol kg}^{-1}$ and remain constant from 38°S to 10°N , then start to increase north of 10°N , presumably due to the influence of the rapidly sinking North Atlantic Deep Water. The value reaches nearly zero at $47^\circ 33'\text{N}$ at a depth of 4,546 m which indicates that the water here is probably not much more than a few years old. The distributions of p_{CO_2} (not shown) look similar to that of $\Delta\Sigma\text{CO}_2^0$.

The computational scheme given above represents only first approximations of the very complicated systems in the oceans. The Redfield-Ketchum-Richards model²⁴ has been assumed to be valid everywhere in the oceans²⁵, and the possible effect of sulphur on TA^{16,17} has not been considered. Waters with the same θ have been assumed to have the same TA⁰ and ΣCO_2^0 and the complicated effects of mixing and the slow vertical diffusive migration of CO_2 from the surface water to deep water regime (T. Takahashi, personal communication) have been neglected. Nevertheless, the method is simple and the results reveal details that have not been shown before. [The work of Brewer was brought to our attention during the preparation of this letter. Although his computational scheme is different from ours, the concepts fall on the same line. His results (his Fig. 2) are similar

to part of our Fig. 2a.] Quantitatively, the results show that the oceanic ΣCO_2^0 has increased by as much as $40 \pm 15 \mu\text{mol kg}^{-1}$ (or $65 \pm 30 \text{ p.p.m. in } p_{\text{CO}_2}$) responding to the atmospheric CO_2 increase. Qualitatively, the results have indicated how far and how deep the human induced CO_2 has penetrated into the Atlantic Ocean. We hope this method can help us to understand better the global carbon cycles.

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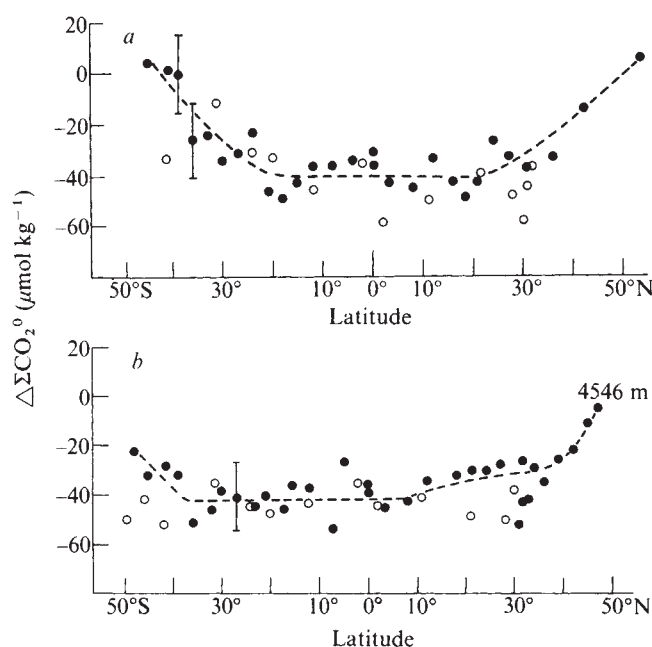


Fig. 2 Variation of $\Delta\Sigma\text{CO}_2^0$ with latitude along the minimum salinity (a) and minimum potential temperature (b) layers in the Atlantic Ocean. ●, Samples taken from the western basin; ○, samples from the eastern basin. The broken lines are drawn through the data in the western basin only.

Particulate uranium, plutonium and polonium in the biogeochemistries of the coastal zone

ALTHOUGH increasing attention has been paid to the role of inorganic solid phases in the chemistry of seawater, little quantitative data has been available to assess their involvement with living systems. We report here recent observations on the uptake of uranium, plutonium and polonium in coastal waters by organisms and submerged surfaces as traced by their isotopes. We show that the body burdens of these radioelements in some marine organisms are governed measurably by the uptake of their particulate forms. Furthermore, these elements are associated with different particulate phases as deduced from the rates at which they deposit on submerged surfaces.

The dissolved uranium content of marine waters seems to be related to the salinity¹. For a salinity of 35‰, the uranium

concentration is $3.35 \pm 0.02 \mu\text{g l}^{-1}$ ($2.48 \text{ d.p.m. l}^{-1}$). Moreover, the $^{234}\text{U}/^{238}\text{U}$ activity ratio is reported as constant at 1.14 ± 0.02 (ref. 2). The disequilibrium of the uranium isotopes in seawater is a consequence of the daughter ^{234}U entering a more readily dissolvable state than its parent, ^{238}U , following its production in crustal rocks. There are several explanations for this phenomenon³. However, the important observation is that the $^{234}\text{U}/^{238}\text{U}$ activity ratio in river water is usually greater than unity, while in the residual solids it is usually of the order of or less than unity. Thus, any continental solid phases that are mobilised to the ocean through the rivers, winds or glaciers may be identified by a $^{234}\text{U}/^{238}\text{U}$ ratio, whose value is <1 . The distribution of plutonium and polonium between soluble and particulate phases in California coastal waters is unknown.

We began exposing solid surfaces (for example, glass beads) to seawater in 1976 to ascertain whether plutonium, which seemed to accumulate on the giant kelps in relation to surface area^{4,5}, would be collected by 'non-living' material. The solids were immersed in seawaters off the Scripps Institution of Oceanography Pier in La Jolla for periods of up to a month, and assayed for plutonium, polonium, and uranium^{2,4}. Independent of the type of material, glass beads, filter paper, alumina balls or PVC, there is a direct relationship between exposure periods and the amounts of uranium, plutonium and polonium picked up from seawater per unit surface area (Fig. 1). Moreover, the uranium ratio of the sorbed phases varies between 0.91 and 1.07, averaging 0.98 ± 0.03 , with probable errors (counting errors) of 0.03 or less.

In the process of attempting to identify the possible organo-metallic complex of uranium, a batch of glass beads was leached with a mixture of chloroform and methanol (2:1). Little of the uranium, plutonium and polonium dissolved in solvent mixture and little was retained on the beads. Instead, these radioactive elements were found in the small amount of particulate material that was released from the beads by the solvent. These elements

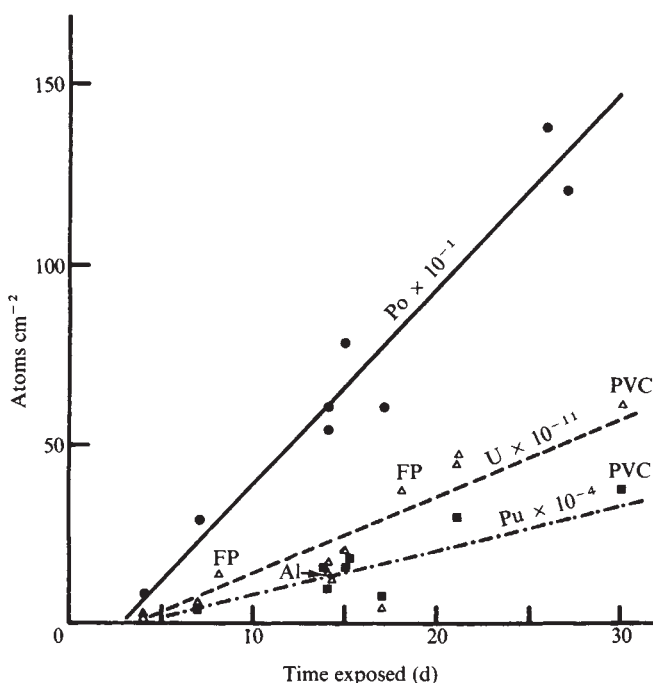


Fig. 1 Accumulations of uranium, plutonium and polonium in atoms cm^{-2} are plotted against the time exposed to seawater off the pier at Scripps Institution of Oceanography, except the 17-d exposure which is on a Navy platform one mile off shore. All points are for the exposure of glass beads except: FP, Whatman 40 filter paper; Al, alumina balls; and PVC, polyvinyl chloride sheet. The accumulation rates are: $\text{U} = 2.3 \times 10^{11} \text{ atoms cm}^{-2} \text{ d}^{-1}$; $\text{Pu} = 1.2 \times 10^4 \text{ atoms cm}^{-2} \text{ d}^{-1}$; and $\text{Po} = 54 \text{ atoms cm}^{-2} \text{ d}^{-1}$.

Table 1 $^{234}\text{U}/^{238}\text{U}$ activity ratio in particulates recovered from seawaters and in seawaters, from Southern California

Particulates collected		$^{234}\text{U}/^{238}\text{U}$	^{238}U	$^{239+240}\text{Pu}$ (d.p.m. l^{-1})	^{210}Po
By filtration (1 μm)	Date				
7,200 l	12/21/77	1.00 ± 0.01	0.0022	0.000024	0.011
22,000 l	1/06/78	0.97 ± 0.01	0.0023	0.000017	0.011
Collected by sedimentation and subsequently resuspended with overnight settling	11/10/77	1.00 ± 0.02			
Collected by one month exposure of acrilan fibres	10/20/77	1.02 ± 0.01			
Seawater					
Volume (l)					
unfiltered					
20	6/02/77	1.14 ± 0.01			
20	7/22/77	1.15 ± 0.01			
Dialysed					
20	9/01/77	1.16 ± 0.01			
8	12/08/77	1.15 ± 0.01			
8	12/19/77	1.15 ± 0.01			

were probably on particles that were fixed to the beads by some type of natural organic adhesive. Thus, submerged bodies, including living algae, may, by an organic coating on their surfaces, collect some fraction of the particles, and the associated inorganic elements, that impact their surfaces in proportion to exposure time and surface area.

It is evident that a portion of uranium, plutonium and polonium in seawater is in particulate form. Of greater interest, however, is the difference in uptake slopes of all three elements on the solid surfaces (Fig. 1). This difference suggests that the elements, although in particulate form, may not be associated with the same solid phases.

Large volumes of seawater were pumped through 1 μm (nominal pore size) glass filters and the particulate material retained by the filters was found to contain concentrations of these elements in ratios roughly equal to those of the glass beads. The U/Po , U/Pu and Pu/Po ratios on an atom basis for the glass beads and seawater particulates were: 4.2×10^9 and 2.4×10^9 , 1.9×10^7 and 2.1×10^7 , 220 and 115 respectively. Moreover, the $^{234}\text{U}/^{238}\text{U}$ activity ratio of the particulates was unity (Table 1). Uranium isotopes were also determined on material that adhered to acrilan fibres exposed for one month in the kelp beds near Scripps and on sediment that collected in an 80 l barrel into which seawater flowed for a period of two months (Table 1). The barrel matter was resuspended in seawater and the fraction that did not settle overnight ($<1-2 \mu\text{m}$ size fraction) was captured by centrifugation at 12,000g. The $^{234}\text{U}/^{238}\text{U}$ ratio of the particulates collected by these two methods was also essentially one (Table 1). In contrast, the total uranium in seawaters from the area has values of the ratio near 1.15 (Table 1). Volumes of seawater dialysed through membranes with molecular weight cut offs of 13,000 and 2,000 gave $^{234}\text{U}/^{238}\text{U}$ ratios of $(1.15-1.16) \pm 0.01$ in the dialysate.

Thus, coastal seawaters near San Diego, California, possess a $^{234}\text{U}/^{238}\text{U}$ activity ratio of 1.15 ± 0.01 , while the particulate phases contained therein and originating on the continents have values around unity. Even though the particulate materials comprise $<1\%$ of the uranium content in the dissolved state, could their impact on the biosphere be quantified? To assess this possibility, the uranium in the soft tissues of filter feeding organisms was assayed for their uranium isotopic composition (Table 2). Brown algae were also analysed.

For most of the biological samples, the $^{234}\text{U}/^{238}\text{U}$ activity ratio is less than that of dissolved uranium (1.15). Note that the soluble uranium activities contributed by the water that constitutes the difference between the wet and dry weights were not subtracted and would only tend to enhance the role of particulate uranium if a correction were made. The largest deviation

from the seawater is in scallops with a value of 0.96 ± 0.01 . On the basis of the uranium isotopic ratio, assuming uranium of dissolved origin has a ratio of 1.15 and particulate uranium a ratio of 0.98, all the uranium found in the scallops was due to the uptake of particulate uranium. Likewise, the observation that the mussels have an average value for the ratio of 1.13 indicates that about 11% of their uranium was taken up as solid phases. The mussels have remarkably high values of uranium in their byssal threads, over 10 times more than in their bodies on the average, and this uranium seems to have arisen primarily from the dissolved state in seawater.

The tunicates, on the other hand, have values of the $^{234}\text{U}/^{238}\text{U}$ ratio markedly lower than those of the mussels. These filter feeders have high hydrogen ion concentrations in their gut regions which could leach uranium from the particulate phases. Both bodies and gut were analysed in three samples. In every case the gut materials had lower values of the ratio but similar values of the uranium concentrations compared to the bodies. Clearly, the particulate phases in seawater contribute significantly to the uranium body burden of these organisms.

The $^{234}\text{U}/^{238}\text{U}$ activity ratio in the algae ranges from 1.09 to 1.16, indicating contributions from both dissolved and particulate uranium. The elk kelp blade (1.16) was thick relative to the macrocystis blades, and thus had less surface area per unit weight for particle deposition. The reverse is seen with the elk kelp scrapings, which maximise the area to weight and have the lowest ratio of the uranium isotopes (1.09)—indicating that 35% of the accumulated uranium was in the particulate form.

Polonium and plutonium are most probably taken up by the algae in a particulate state, by impact as opposed to active

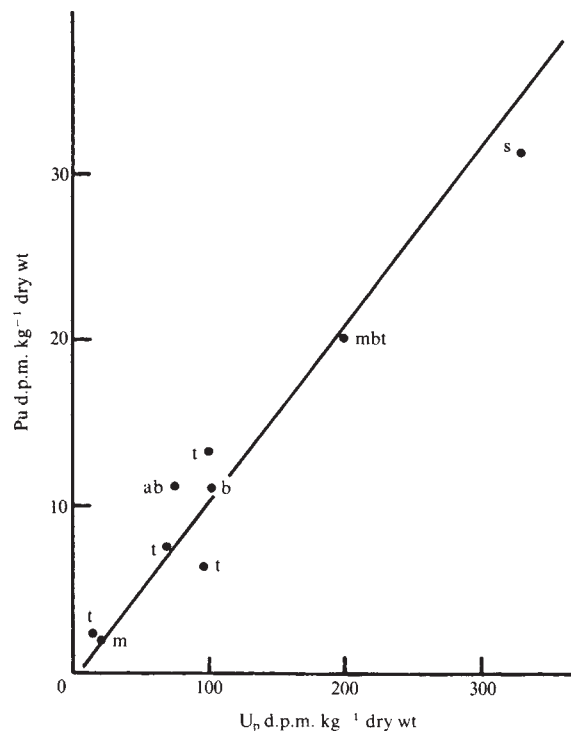


Fig. 2 The activity of plutonium and particulate uranium U_p are linearly proportional with a slope of 1/10. s, scallops; mbt, mussel byssal threads; t, tunicates; ab, abalones; b, barnacles; and m, mussels.

Table 2 Uranium concentrations and isotopic composition in marine organisms from California coast (on a dry weight basis)

	^{238}U (d.p.m. per kg)	$^{234}\text{U}/^{238}\text{U}$ activity ratio
Algae		
Elk kelp (large blade)	102 ± 1	1.16 ± 0.02
Elk kelp (surface scrapings)	—	1.09 ± 0.02
Macrocystis	358 ± 4	1.13 ± 0.01
Macrocystis	508 ± 2	1.10 ± 0.01
Mussels (<i>Mytilus</i> sp.)		
Pt Fermin	130 ± 1	1.12 ± 0.01
Pt Arguello	118 ± 1	1.13 ± 0.01
Bodega Head	121 ± 1	1.13 ± 0.01
Oceanside	224 ± 1	1.09 ± 0.01
San Simeon	138 ± 1	1.12 ± 0.01
Diablo Canyon	200 ± 1	1.15 ± 0.01
Santa Cruz	177 ± 1	1.11 ± 0.01
Soberanas Pt	241 ± 1	1.15 ± 0.01
N San Francisco	251 ± 1	1.15 ± 0.01
S San Francisco	172 ± 1	1.10 ± 0.01
Farallon Is	246 ± 1	1.15 ± 0.01
San Diego	125 ± 1	1.13 ± 0.01
Byssal threads		
Bodega Head	$9,100 \pm 40$	1.12 ± 0.01
Cape Flattery	$3,860 \pm 30$	1.15 ± 0.02
Farallon Islands	$4,640 \pm 20$	1.13 ± 0.01
Tunicates		
<i>Polycrinum planum</i>	140 ± 1	1.07 ± 0.01
	63 ± 1	1.12 ± 0.01
<i>Styela monterensis</i>	body-1	116 ± 1
	gut-1	149 ± 1
	body-2	207 ± 1
	gut-2	245 ± 1
<i>Ciona intestinalis</i>	body	245 ± 1
	gut	330 ± 1
Abalone	760 ± 1	1.13 ± 0.01
Gooseneck barnacle	206 ± 1	1.07 ± 0.01
Barnacle gills + intestine	169 ± 1	1.13 ± 0.02
Scallops	329 ± 1	0.96 ± 0.01
Rock oysters	278 ± 1	1.07 ± 0.05

processes. The Pu/Po atom ratios on the glass beads and kelp are 220 and 280 respectively. The ratio for seawater particulates is 115, also roughly the same. Even more remarkable is that the rates of uptake of polonium and plutonium in atoms $\text{cm}^{-2} \text{d}^{-1}$ by the glass beads and the kelp are essentially identical, $\text{Po} = 54$ and 52 (ref. 4) and $\text{Pu} = 1.2 \times 10^4$ for both the beads and kelp⁴.

The plutonium concentrations in the other marine organisms are linearly proportional to the uranium of particulate origin (Fig. 2). Thus, the uptake of plutonium by these organisms may be related to the ingestion of this transuranic in a particulate form.

Clearly, uptake of particulates can significantly influence the uranium, polonium and plutonium content and the uranium isotopic ratio of coastal marine organisms. By simply measuring the uranium isotopic ratios, the extent to which particulate uranium is important can be quantitatively determined. Using the same approach, it may be possible to assess the role of particulate trace elements by comparing their specific activities, for example, ^{210}Pb /stable lead or $^{238}\text{Pu}/^{239+240}\text{Pu}$ on particulate phases in seawater and in the organisms.

These studies also point out that caution must be exercised in calculating a concentration factor for trace elements in organisms over the concentration of that element in seawater. For example, the scallops accumulate particulate uranium exclusively and, therefore, using the ^{238}U particulate concentration in seawater of $0.002 \text{ d.p.m. l}^{-1}$ (Table 1), the concentration factor based on the dry weight of the organisms is 1.5×10^5 . Had the total ^{238}U seawater concentration been used to calculate this factor, a value of roughly 150 would have been reported. Note that the build up on the beads after one month of exposure to seawater for uranium, plutonium and polonium is $\sim 1 \times 10^7$, using the fact that 4.5 kg of beads collect nearly 1 g of dry particulates. On the other hand, the byssal threads of the mussels from Bodega Head, California, have a concentration factor for soluble uranium of 3.3×10^3 and one for insoluble uranium of 7.4×10^5 .

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¹⁰Be in marine sediments, Earth's environment and cosmic rays

COSMIC RAYS create the long-lived radioactive nuclide ¹⁰Be (half life: 1.5 M yr) continuously in the upper atmosphere as a result of nuclear disintegrations. It enters the oceans through various paths and finally deposits in deep-sea sediment in a time which is short compared to its half life. This isotope has been used to establish the chronology of geophysical events up to the Pliocene¹⁻⁷ or to suggest cosmic-ray intensity variations in the past^{8,9}. This report shows that the ¹⁰Be stratigraphy in marine sediments can be used as a time marker for geophysical events in the past, but not for delineating any time variations in cosmic-ray intensity unless one can learn how to delineate geophysical effects. Also the cosmic-ray consistency would be estimated from the limits of ¹⁰Be variation implied by this data set.

A sediment core (KH68-4-18), 968 cm in length, was collected from a 5,390 m deep-ocean basin at 01° 59' N, 170° 01' W, central equatorial Pacific. Beryllium was separated from the

sediment samples using an improved chemical method¹⁰, and the β -ray activity of ¹⁰Be was counted with two sets of low-level needle counters¹¹ and a low-level silicon detector; background count rates and counting efficiencies of these were $0.0082 \pm 0.0005 \text{ min}^{-1}$, $51 \pm 3\%$, $0.0072 \pm 0.0005 \text{ min}^{-1}$, $51 \pm 3\%$, $0.0372 \pm 0.012 \text{ min}^{-1}$, $35 \pm 2\%$ (90-555 keV), respectively.

The absolute age $T(x)$ at a depth x was evaluated¹² from a rate of ¹⁰Be participation, P (atoms $\text{cm}^{-2} \text{ s}^{-1}$), onto sediment surface and the specific activities of ¹⁰Be, $N(x)$ (d.p.m. cm^{-3} or atoms cm^{-3}), in all successive layers along the core down to a depth, x .

$$N(x) = \frac{P}{S(x)} \exp[-\lambda T(x)], \quad dT(x) = \frac{dx}{S(x)}$$

or

$$T(x) = -\frac{1}{\lambda} \ln \left[1 - \frac{\lambda}{P} \int_0^x N(x) dx \right]$$

Here, λ is a decay constant of ¹⁰Be and P is assumed to be constant and equal to $2.15 \times 10^{-2} \text{ }^{10}\text{Be atoms cm}^{-2} \text{ s}^{-1}$, during the time interval considered for this core, and the sedimentation rate at a layer, $S(x)$, is assumed to have varied with depth or time.

The specific activities of ¹⁰Be and the ¹⁰Be ages are listed in Table 1 together with magnetic ages¹³, differential sedimentation rates and the content of Be and Mn. The uncertainty in the specific activities was estimated on the basis of the uncertainties (1σ) in chemical determination of beryllium (8%), counting efficiency (5%), absorption correction (5%) and counting statistics (7-17%).

The ¹⁰Be ages agree well with the magnetic ages at six reversal horizons (Table 1). The magnetic reversal pattern of core KH68-4-18 is very clear and consistent with palaeontological analysis¹³. These agreements show that the assumption of constant ¹⁰Be precipitation would be correct and the cosmic-ray production rates integrated over the time intervals ($2-7 \times 10^5 \text{ yr}$) between each adjacent reversals have been constant ($< \pm 10\%$

Table 1 Specific activities of ¹⁰Be, ¹⁰Be ages, magnetic ages, differential sedimentation rates and concentration of chemical elements in core KH68-4-18

Depth interval (cm)	Dry weight of sample (g)	In situ density* (g cm ⁻³)	Specific activity of ¹⁰ Be (10 ⁻³ d.p.m. cm ⁻³)	¹⁰ Be age (Myr)	Magnetic age† (Myr)	Differential sedimentation rate (mm kyr ⁻¹)	Chemical elements Be (p.p.m.)	Mn (%)
0-35	9.7	0.24	1.77 ± 0.23	0.11		3.2	1.85	0.55
35-75	14.2	0.24	1.57 ± 0.20	0.22		3.6	1.78	0.53
75-110	12.4	0.25	1.20 ± 0.17	0.30		4.4	1.65	0.32
110-155	13.7	0.24	1.25 ± 0.15	0.41		4.1	1.66	0.55
155-190	12.1	0.25	1.25 ± 0.18	0.50		3.9	2.24	0.56
190-230	13.8	0.24	0.97 ± 0.13	0.59		4.4	1.49	0.54
230-265	11.6	0.24	1.37 ± 0.20	0.69	0.69	3.5	1.88	0.56
265-300	10.0	0.24	1.09 ± 0.15	0.78		3.9	1.75	0.60
300-335	11.7	0.23	1.05 ± 0.15	0.87	0.89	3.9	2.15	0.46
335-365	9.8	0.23	1.19 ± 0.20	0.97	0.95	3.0	1.52	0.44
365-415	18.8	0.25	1.21 ± 0.17	1.13		3.1	1.72	0.56
415-465	18.9	0.26	0.62 ± 0.09	1.23		5.0	1.70	0.66
465-515	17.9	0.24	1.34 ± 0.18	1.43		2.5	1.82	0.64
515-570	18.3	0.24	1.01 ± 0.13	1.61	1.65	3.1	1.76	0.61
570-625	12.0	0.23	1.14 ± 0.16	1.85	1.85	2.3	1.64	0.51
625-675	16.7	0.22	0.76 ± 0.10	2.00		3.3	1.79	0.52
675-730	17.2	0.22	0.75 ± 0.12	2.19		2.9	1.87	0.50
730-790	22.5	0.24	0.69 ± 0.13	2.39		3.0	1.70	0.44
790-850	20.1	0.23	0.14 ± 0.08	2.43	2.43	15.0	1.56	0.37
850-905	18.6	0.23	0.34 ± 0.06	2.53		5.5	1.59	0.53
905-968	18.5	0.23	0.86 ± 0.16	2.84		2.0	1.53	0.46

* In situ density is defined as the ratio of the dry weight of sediment to the in situ volume occupied by the sediment.

† The depth is measured from the top of clay sediment filled in core pipe, whereas the depth presented in ref. 5 where the magnetic age has been determined is measured from the top of core pipe; the difference is 15 cm. The magnetic age tabulated here is normalised to the present depth.

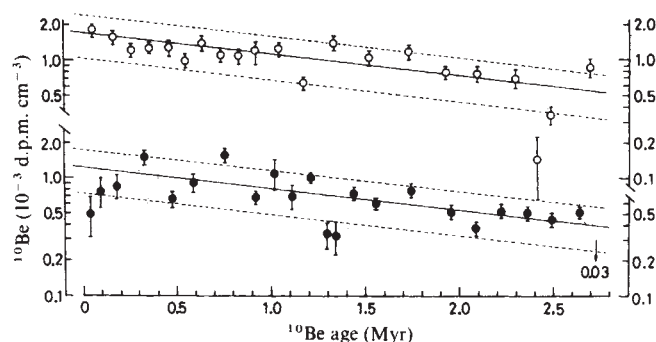


Fig. 1 Comparison of the ^{10}Be variations with time in two Pacific deep-sea sediment cores: ○, KH68-4-18; ●, KH68-4-25. Data for core KH68-4-25 taken from ref. 12. Mean decay lines are shown by solid lines, with $\pm 40\%$ limits of variation by dotted lines.

variation) up to 2.5 Myr BP. The differential sedimentation rates at different layers vary by a factor of 2 from $\sim 2.5 \text{ mm kyr}^{-1}$ to $\sim 5 \text{ mm kyr}^{-1}$ except for 15 mm kyr^{-1} at a layer 790–850 cm. The mean sedimentation rate is 3.5 mm kyr^{-1} consistent with the value of 3.4 mm kyr^{-1} (ref. 13) derived from magnetic stratigraphy.

The ^{10}Be activities in core KH68-4-18 (2°N , 170°W) and a previously investigated¹² core KH68-4-25 (20°S , 170°W) are plotted against the ^{10}Be ages in Fig. 1.

The ^{10}Be activities in the two cores do not parallel each other in the past. This fact rules out cosmic-ray intensity changes as the principal cause for the ^{10}Be variations. Another fact unfavourable to the simple cosmic-ray interpretation is that the rate of ^{10}Be precipitation at 2°N ($2.15 \times 10^{-2} \text{ atoms cm}^{-2} \text{ s}^{-1}$) is twice as large as that at 20°S ($1.14 \times 10^{-2} \text{ atoms cm}^{-2} \text{ s}^{-1}$, see ref. 12). If the ^{10}Be precipitation rate reflects the latitudinal variation of the overhead ^{10}Be fallout¹⁴, the P values for the two cores would be reversed.

Non-parallelism of the ^{10}Be variations in the two cores is also unfavourable to a simple global climate change as a main cause for the ^{10}Be variations¹⁵, although the glacial and interglacial effects in principle could influence the ^{10}Be content of ocean sediments in a relatively complex fashion.

One of the most significant differences between the two cores is the low ^{10}Be concentrations (Recent 0.27 Myr) at the top of core KH68-4-25, that do not occur for core KH68-4-18. Tanaka *et al.*^{12,16} showed that the volcanism of Samoan island arc caused the decrease at the top of core KH68-4-25, on the bases of the abundance of volcanic glassy fragments and the variation of elemental abundances. In core KH68-4-18, there are no glassy fragments of volcanic origin and no correlation between the concentrations of ^{10}Be and elemental abundances; volcanism has little effect for this core. This equatorial core has plenty of fossils of faunal and floral species throughout (30–80% in volume under microscope), with exceptions at depth 415–465 cm (1.13–1.23 Myr) and 790–850 cm (2.39–2.43 Myr) where the fossil occurrences are sporadic (less than a few %) and the ^{10}Be concentrations are low (see Fig. 1). Biological productivity may cause changes in ^{10}Be concentrations.

Another set of analysis will be stated on the limits of ^{10}Be variation implied by this data set. The data show some scatter in the plot of $\log N(x)$ versus time (Fig. 1), but the decay line (solid lines in Fig. 1) having the slope of a half life of 1.5 Myr which starts from the $N(0)$ value derived from the adopted P value and mean sedimentation rate seems to be representative for the data set. The limits of ^{10}Be variation from the mean line are $\pm 40\%$ for both cores, represented by dotted lines in Fig. 1. A few points beyond the limits can be explained by special effects such as the presence of volcanic fragments or the low biological productivity. The ^{10}Be variations are mainly affected by complex environmental factors, and the cosmic-ray intensity variations, if

any, do not exceed at most $\pm 40\%$ for period of $\sim 1 \times 10^5 \text{ yr}$ (sampling intervals) during the past 2.5 Myr.

The ^{10}Be variations with time and with oceanic location reflect complex changes in the Earth's environment such as volcanism, biological productivity, oceanic circulation, and climate. The cosmic-ray intensity variations, if any, do not exceed $\pm 10\%$ for periods of $2 \sim 7 \times 10^5 \text{ yr}$ and $\pm 40\%$ for periods of $\sim 1 \times 10^5 \text{ yr}$ during the past 2.5 Myr, and can not be delineated from this data set.

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Human chimaera detectable only by investigation of her progeny

A CHIMAERA can be defined as an organism whose cells derive from two or more zygotes¹. In man, two categories of spontaneous and permanent chimaeras are recognised: blood chimaeras which arise from the exchange of blood-forming cells between dizygotic twins, and dispermic chimaeras which are the result of the fusion of two zygotes and of their growth into one body (for review see ref. 2). Blood chimaeras are usually identified because of blood-grouping problems caused by the mixture of different erythrocyte populations, while dispermic chimaeras who often present sex abnormalities are mainly detected by cytogenetic studies, although they also carry a mixture of different blood cell populations. In contrast to these typical cases, we now report a unique human chimaera who shows no mixture of blood cells as far as the genetic markers investigated are concerned, and who is only detectable by the investigation of the blood of her progeny (family Eiw., Fig. 1).

This chimaera was brought to our attention because of an apparent mother-child exclusion in the ABO system: mother II.2: A₂B, child III.4: O. Blood samples of the mother II.2, her husband II.1, her children III.1–III.4 and her parents I.1 and I.2 were typed for the following markers: ABO, rhesus, MNSS, P, Kell, Duffy, Kidd, Lutheran, Lewis, ABH- and Lewis-secretion, Xg, acid phosphatase acP-1, phosphoglucomutase PGM-1 (including the subtypes, PGM-1^a, defined by isoelectric focusing on polyacrylamide gel³), adenylate kinase AK-1, adenosine desaminase ADA, glutamate pyruvate transaminase GPT, glyoxalase GLO-1, esterase D EsD, gammaglobulin groups Gm

Table 1 Genetic marker systems showing mother-child exclusions (family Eiw.)

	Born	ABO	Rhesus	Kidd	AcP-1	PGM-1 ^a	Bf	HLA
I.1	1896	B	CcDee	a+b+	B	NT*	S	A1, 2; B7, 37
I.2	1901	A ₂	CcDee	a+b+	AB	a3-a1	FS	A3, w31; Bw35, w52; Cw4
II.1	1924	B	ccdde	a+b+	B	a1	S	A3, 26; B15, w38; Cw3
II.2	1937	A ₂ B	ccdde	a-b+	B	a3-a2	S	A2, w31; B7, w52
III.1	1967	B	ccdde	a+b-†	B	a2-a1	FS‡	A3, 26; Bw35, w38; Cw4†
III.2	1969	O†	ccdde	a+b+	AB‡	a1†	FS‡	A3, 26; Bw35, w38; Cw4†
III.3	1970	B	CcDee‡	a-b+	AB‡	a1†	FS‡	A3; B15, w35; Cw3, w4†
III.4	1975	O†	CcDee‡	a-b+	B	a1†	FS‡	A3, 26; Bw35, w38; Cw4†

* PGM-1^a not tested, PGM-1 phenotype of I.1 in starch gel electrophoresis: 2-1.

† Mother-child exclusions without taking into account the father II.1.

‡ Mother-child exclusions taking into account the father II.1.

and Km, haptoglobins Hp, group specific components Gc, prothrombin factor B Bf and HLA.

It became evident that all four children III.1-III.4 did not fit genetically with their mother II.2 whilst there was no evidence for an exclusion of the father II.1. The systems showing mother-child exclusions without taking into account the father II.2 are listed in Table 1: III.1 is excluded by Kidd and HLA, III.2 and III.4 by ABO, PGM-1^a and HLA and III.3 by PGM-1^a and HLA. Accepting the paternity of II.1, the mother is additionally excluded from III.1-III.4 by Bf, from III.2 and III.3 by acP-1 and from III.3 and III.4 by rhesus (Table 1). Thus, the mother-child exclusions include genes governed by chromosome 1 (rhesus and PGM-1^a)^{4,5}, chromosome 2 (acP-1)⁶, chromosome 6 (Bf and HLA)^{7,8}, chromosome 9 (ABO)⁹, and possibly chromosome 7 (Kidd)¹⁰. Significantly, all the genes (or haplotypes) which must have been transmitted from the mother II.1 to her four children and which are not detectable in her blood can be found in the maternal grandparents: *O* potentially present in I.1 and in I.2, *CDe* and *Jk^a* in I.1 and I.2, acP-1 *P^a*, *Bf^F* and *HLA-A3, Bw35, Cw4* in I.2, and PGM-1^a *a¹* in I.2 and possibly in I.1. The four children III.1-III.4 are therefore genetically compatible with their father II.1 on the one hand and their maternal grandparents I.1 and I.2 on the other hand.

The results of unidirectional mixed lymphocyte cultures (Table 2) showed that I.2 is able to stimulate her daughter's (II.2) lymphocytes, although II.2 carries obviously in her organism both HLA haplotypes of her mother. The stimulation observed is in good agreement with a one HLA haplotype difference between both individuals. This lack of immunological tolerance demonstrates that all the cells of II.2 which express HLA-D gene products (lymphocytes, monocytes¹¹, epithelial¹² and endothelial cells¹³) do not possess the HLA haplotype *A3, Bw35, Cw4*.

Fibroblasts of II.2 have been cultivated from skin biopsies of both forearms. The typing results of all the markers tested for in these cells (acP-1, PGM-1^a, EsD, GLO-1 and HLA) were identical to the findings obtained using blood cells.

The karyotypes of all family members were analysed using Q-, G- and C-banding techniques. There was no evidence for numerical or structural abnormalities and, checking chromosome polymorphisms, no mother-child exclusion could be found. The mother II.2, in particular, showed in her lymphocytes and in her fibroblasts the normal female karyotype 46, XX.

Table 2 Results of unidirectional mixed lymphocyte cultures (MLC) in family Eiw. expressed in c.p.m. of ³H-thymidine uptake

Responders	Stimulators (X-irradiated)		
	I.2 _x	II.2 _x	UR _x *
I.2	716	17,154	60,279
II.2	9,200	1,319	26,007
UR*	55,783	33,825	951

The numbers represent the mean value of ³H-thymidine uptake in triplicate tests.

* Unrelated control person.

An interchange of all four children, *a priori* improbable, could be ruled out by interrogating the staff of the obstetric department in which the four children had been born. Furthermore the mother II.2 has no twin sibling and does not show in her circulation more than one erythrocyte and/or lymphocyte population.

The findings in this family clearly show that II.2 possesses in her organism at least two populations of genetically different cells, one demonstrable in her blood as well as in the fibroblasts, and the other one in her gonads; therefore, she must be considered a chimaera. Although the analysis of the genetic markers carried by the different cell populations of II.2 gives no evidence for the involvement of two sperms, the most simple explanation of this observation is to assume a dispermic chimaerism. The cells with the HLA haplotype *A3, Bw35, Cw4* present in the gonads of II.2 must have a rather restricted tissue distribution, as the cells of ectodermal and mesodermal origin studied do not carry the HLA-D determinant coded for by this haplotype.

Thus this family demonstrates the existence of chimaeras who do not show different populations of erythrocytes, lymphocytes and fibroblasts and can be detected only by the analysis of their progeny. Such individuals must be extremely rare, but the practical implications of this observation for problems of parentage are clear.

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The *Drosophila* memory mutant *amnesiac*

POPULATIONS of *Drosophila melanogaster* can be taught to avoid specific odours which they have experienced in conjunction with electric shock^{1,2}, and several single-gene mutants have been isolated which fail to learn this task (ref. 3, and our unpublished data). Here we describe a mutant, *amnesiac*, which learns normally but forgets four times as quickly as wild-type flies.

Stocks, culture methods, apparatus and testing conditions are described in ref. 1. Male *D. melanogaster* of the Canton-Special (C-S) wild-type strain were treated with the mutagen ethyl methane sulphonate⁴, and progeny populations were bred from them using the attached-X method⁵. This yield stocks of flies in which the males have identical, mutagenised X chromosomes. Males from each stock were selected under CO₂ anaesthesia and retained for testing 1–3 days later. To screen for mutants with abbreviated memory retention, we trained flies from each mutagenised population by exposing them 10 times to a chemical odorant, 3-octanol, in association with shock⁵, then left them undisturbed for 45 min to give them an opportunity to forget. The flies were tested by running them alternately towards (1) a tube containing 3-octanol (O), the odorant they had been taught to avoid, and (2) a tube containing 4-methylcyclohexanol (M) a novel, control odorant. Figure 1 shows the number of flies which avoided each. To control for odour bias, flies which had been trained to avoid M were tested in the same apparatus. The index of learning, Λ , is the fraction of the population avoiding the shock-associated odour, minus the fraction avoiding the control odour, averaged for the two halves of the experiment. (All Λ values reported here are mean $\pm$ s.e.m. for 10 experiments.) In these conditions, wild-type flies learn and retain selective avoidance for 45 min. Seven hundred mutagenised stocks were tested; one, *amnesiac* (*amn*^{PS801}) gave reproducibly poor performance in this paradigm.

The *amnesiac* males selected above were used to breed a pure stock of mutant males and females. Male *amn* flies were mated to females carrying the X-chromosome balancer FM7a (ref. 6)

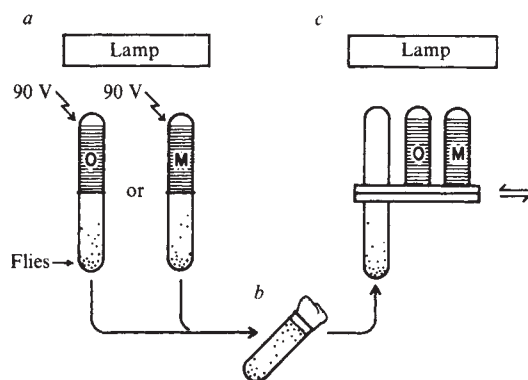


Fig. 1 Measuring memory retention of mutagenised stocks. *a*, Training: a population of 30–50 flies was taught to avoid a specific odour. The light attracted the flies from the start tube into a contiguous tube containing a chemical odorant (either 1 μ l 3-octanol (O) or 2 μ l 4-methylcyclohexanol (M), spread on an electrified grid (60 Hz, 90 V a.c.)). After 60 s, the flies were shaken back into the start tube and another training run was begun. This cycle was repeated for 10 runs. *b*, Waiting: flies were left undisturbed for 45 min in dim light at room temperature (22 °C). *c*, Testing: the sliding counter-current apparatus¹ allowed the fresh start tube, with flies, to be shifted opposite either of two (non-electrified) grids, one spread with O, the other with M. The flies were shifted opposite the O grid and given 15 s to run, after which the flies remaining in the start tube were counted. After 45 s rest in the 'rest' tube (without grid), they were shifted opposite the M tube and their avoidance was similarly scored.

Table 1 Latent memory in normal and *amnesiac* flies

	Λ_1 (flies trained twice to same odour)	Λ_2 (flies trained to opposite odours)	$\Lambda_1 - \Lambda_2$
No anaesthesia			
C-S	0.24 $\pm$ 0.02	0.01 $\pm$ 0.03	0.23 $\pm$ 0.04
<i>amnesiac</i>	0.28 $\pm$ 0.03	0.03 $\pm$ 0.01	0.24 $\pm$ 0.04
Anaesthesia 30 min after first training session			
C-S	0.20 $\pm$ 0.03	0.07 $\pm$ 0.01	0.13 $\pm$ 0.03
<i>amnesiac</i>	0.17 $\pm$ 0.02	0.04 $\pm$ 0.01	0.13 $\pm$ 0.03

Populations were trained 10 times to avoid O or M as in Fig. 1, then left undisturbed for 45 min. Flies were then given a single cycle of training¹, which consisted of a 30-s run towards an odour (O or M) with shock, a 30-s wait, and a 30-s run towards the control odour (M or O) without shock. Thirty seconds later the flies were tested as usual. The four possible combinations of two aversive training sessions were run 10 times each. Scores from flies trained against the same odour in both sessions (O $\rightarrow$ O and M $\rightarrow$ M) were averaged to give Λ_1 values. Similarly, scores from flies trained against opposite odours (O $\rightarrow$ M and M $\rightarrow$ O) give Λ_2 values. Learning indices are calculated relative to the last training sessions: positive difference between the two Λ values indicates that the earlier training session affected behaviour. Such a difference is present for both normal and *amnesiac* flies. Moreover, in both genotypes, the difference persists when the flies are briefly anaesthetised 30 min after the first training sessions. Therefore cryptic memory, present in *amnesiac* flies, can be consolidated to the long-term (anaesthesia-resistant) form.

and C-S autosomes. Female FM7a/*amn* progeny were mated to *amnesiac* males, yielding F₂ progeny from which a pure stock of hemizygous *amn* males and homozygous *amn/amn* females could be selected by the absence of *Bar*, a dominant visible marker on the FM7a chromosome. All subsequent experiments on *amnesiac* were done with mixed males and females, 3–5 days old.

The 10-trial training procedure above is a modification of the original learning paradigm to give enhanced memory retention. It was used only for mutant screening and in genetic mapping and latent memory experiments as specified below. All other memory measurements were made using the basic olfactory paradigm of ref. 1. This is similar to the procedure in Fig. 1 except that it uses 3 training trials instead of 10, and during training, flies are alternately exposed to both O and M (one with shock, one without), to provide an internal control for sensitisation. In this olfactory paradigm the expression of learning is statistical; even in our best conditions only a third of the flies show learning in any given trial. This limits our understanding of what individual flies in the population are doing. However, we have previously found that normal C-S flies appear to be homogeneous in their capability to learn; the selective avoidance observed is not due to an 'intelligent' subset of the population¹. A likely reason for our relatively low learning scores is that the task we have chosen—olfactory discrimination and active avoidance—is a difficult one for *Drosophila*. In fact, the flies learn much better in a simpler paradigm, as that originally developed for cockroaches by Horridge⁸. Of 25 normal individuals tested 24 (96%) consistently lifted their legs to avoid shock (data not shown).

Populations of *amnesiac* and normal C-S flies were trained in the olfactory paradigm, left undisturbed for various intervals, then tested to obtain the memory decay curves in Fig. 2. Immediate learning in the two stocks is similar, but memory in *amnesiac* flies decays rapidly and is undetectable 60 min later (avoidance of both shock-associated and control odours decreases, so that 60 min after training the behaviour of *amnesiac* is undistinguishable from that of untrained flies). In contrast, memory in C-S persists for 6 hours in these conditions².

Some uninteresting explanations for *amnesiac*'s forgetfulness have been ruled out. The decline in the mutant's performance is not due merely to lethargy at testing time. Populations were

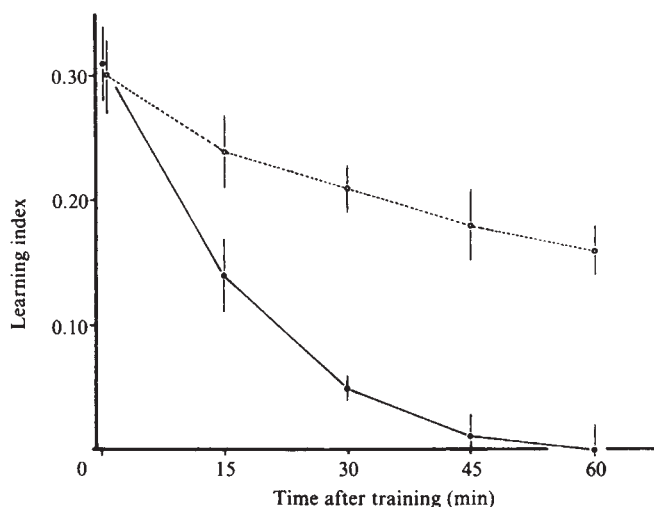


Fig. 2 Memory retention of normal (dashed line) and *amnesiac* (solid line) flies. Populations of C-S and *amnesiac* flies were given three training trials, left undisturbed for the periods indicated, then tested (procedure as in ref. 1, except that flies were shifted to fresh tubes just before testing). The two genotypes were trained and tested simultaneously.

trained, left for 45 min, then given brief (30-s) electric shock, in an attempt to re-invigorate them, and tested. The mutant still failed to demonstrate memory [$\Lambda(amn) = 0.03 \pm 0.03$, $\Lambda(C-S) = 0.15 \pm 0.02$]. To find out whether the memory decline is a result of a general deterioration in *amnesiac*'s capabilities brought on by trauma in training, flies were trained, left for 60 minutes, trained again (half to the same odour, half to the other) and immediately tested. The mutant still had normal capacity for learning ($\Lambda = 0.32 \pm 0.02$).

The sensory and motor abilities of *amnesiac* appear normal. Phototaxis as measured by countercurrent distribution⁷ is similar to C-S. Mutant flies show normal avoidance of electrified grids, jump when shocked, and have normal leg-to-leg resistance of about $10^9 \Omega$ (ref. 3). The sensitivity of *amnesiac* to the odour cues used is also similar to the parent strain. Normal flies have a slight intrinsic tendency to avoid the odourants O and M, which is overcome in the learning paradigm by strong phototactic drive toward the odour tubes. This aversion can be used to

measure the flies' olfactory capability in a 'choice chamber'—a T-maze in which flies are passively transported to the choice point. One arm contains a defined amount of odourant; the other is left blank³. In this test, the odour avoidance behaviour of normal and *amnesiac* flies was indistinguishable. The fraction of the population which avoided O (2 μ l per tube) was 0.83 ± 0.03 for *amnesiac*, and 0.81 ± 0.04 for C-S. The fraction avoiding M (2 μ l per tube) was 0.65 ± 0.06 for *amnesiac*, and 0.70 ± 0.03 for C-S.

We also measured *amnesiac*'s memory using other odour cues. Flies were trained and tested as in Fig. 2, except that amylacetate and benzaldehyde² (both 2 μ l per grid) were substituted for O and M. As before, the mutants learned normally ($\Lambda(amn) = 0.31$, $\Lambda(C-S) = 0.30 \pm 0.01$) but forgot quickly; 45 min after training $\Lambda(amn) = 0.02 \pm 0.01$, while $\Lambda(C-S) = 0.15 \pm 0.02$.

Drosophila, like higher animals, has separate memory storage phases—short-term memory, which is sensitive to cold anaesthesia, and long-term memory, which is not^{2,5}. It seemed possible that the *amnesiac* mutation acted by blocking consolidation into long-term memory. Testing this idea directly is impossible, because memory in the mutant is virtually undetectable by the time consolidation would normally take place. Therefore, to lengthen *amnesiac*'s memory span, we increased the number of training trials and adopted a procedure akin to the psychological technique of 'savings measurement', in which memory can interact with more recent learning.

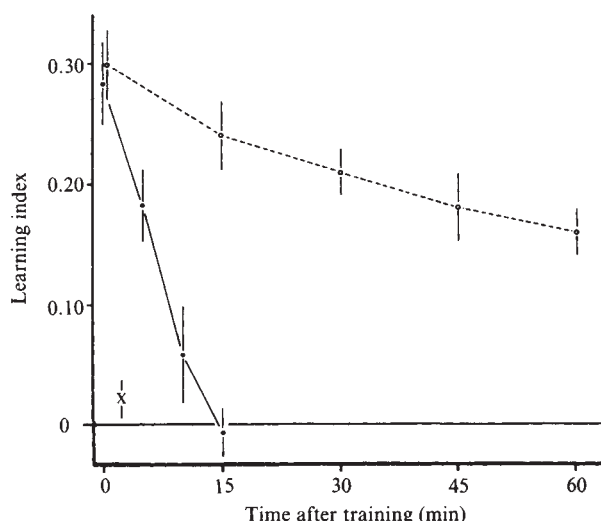


Fig. 3 Memory retention in normal flies (dashed line), *turnip* mutants (X), and *turnip*/+ heterozygotes (solid line). Method as in Fig. 2. The heterozygotes did not regain their memory later; learning indices at 20 and 60 minutes remained near zero.

We shocked flies 10 times in conjunction with either O or M, left them undisturbed for 45 min to forget, then gave them a single training run to one of the odours and tested them. All four possible combinations of the two training sessions were run, that is: O → O, M → M, O → M and M → O. If latent memory from the first training session were present to interact with learning from the final training cycle, populations which had been trained to avoid the same odour in both sessions (O → O and M → M) should show higher learning scores than populations trained in opposite directions (O → M and M → O). Table 1 shows that this was the case, for both normal and *amnesiac* flies. Moreover, for both genotypes, the effect of the first training session was largely resistant to brief cold anaesthesia applied 30 min later. The *amnesiac* mutants, like normal flies, can convert their memory into anaesthesia-resistant form; so the mutation does not block consolidation into long-term memory.

More surprisingly, Table 1 shows that in these conditions the earlier training has quantitatively similar behavioural effects in normal and *amnesiac* flies. Apparently, memory is present in

Table 2 Mapping the *amnesiac* mutation

Class	Memory (Λ) 45 min after training
Parental chromosomes:	
<i>amnesiac</i>	0.04 ± 0.01 ($n = 14$)
<i>sc ec cv ct⁶ v g² f</i>	0.19 ± 0.03 ($n = 27$)
Recombinant chromosomes:	
<i>ec cv ct⁶ v g² f</i>	0.21 ± 0.06 ($n = 9$)
<i>cv ct⁶ v g² f</i>	0.15 ± 0.02 ($n = 9$)
<i>ct⁶ v g² f</i>	0.10 ± 0.01 ($n = 8$)
<i>v g² f</i>	0.14 ± 0.01 ($n = 16$)
<i>g² f</i>	0.15 ± 0.03 ($n = 14$)
<i>f</i>	0.17 ± 0.02 ($n = 17$)
<i>sc</i>	-0.01 ± 0.03 ($n = 10$)
<i>sc ec</i>	0.06 ± 0.02 ($n = 12$)
<i>sc ec cv</i>	0.10 ± 0.02 ($n = 8$)
<i>sc ec cv ct⁶</i>	0.04 ± 0.03 ($n = 9$)
<i>sc ec cv ct⁶ v</i>	0.03 ± 0.03 ($n = 10$)
<i>sc ec cv ct⁶ v g²</i>	0.05 ± 0.03 ($n = 9$)

Memory scores, 45 min after training, are shown for parental chromosomes and each recombinant class. Each chromosome was tested over *amnesiac* in heterozygous females, to eliminate effects of markers. Recombinant chromosomes with the *forked* marker have high memory scores; chromosomes with *forked*⁺ have low scores. This places the location of the *amnesiac* marker very close or to the right of *forked*, near the centromere of the X chromosome.

the mutant. It is cryptic after the first training session, inaccessible to direct expression ($\Lambda = 0.02 \pm 0.02$) but can interact with more recent learning to affect behaviour just as in normal flies. This suggests that the *amnesiac* mutation affects memory retrieval rather than storage.

Genetically, *amnesiac* behaves as a sex-linked recessive. Memory of *amn*/+ heterozygotes 45 min after training was normal ($\Lambda = 0.18 \pm 0.03$). The mutation was mapped on the X chromosome using methods similar to those of Dudai *et al.*³. Female *amnesiac* flies were crossed with males carrying the visible X-linked markers *scute*, *echinus*, *cut*⁶, *vermillion*, *garner*², *forked*. Recombinant F₂ males were collected and scored for the visible markers. These could not be tested directly for the presence of *amnesiac* because the markers affect the performance in learning tests. Therefore, recombinants were individually mated to *amnesiac* females, producing female progeny in which the recessive visible markers were heterozygous, and not expressed. These F₃ females are homozygous for *amn* if the mutation was present in the F₂ male; if the females forget rapidly one can infer that *amn* was present in the original recombinant. Table 2 shows memory scores of progeny from the various recombinant types. It can be seen that the *amnesiac* mutation maps near or to the right of *forked* (1–56.7) on the X chromosome. Preliminary data from a second cross suggest that *amn* lies between *forked* and *carnation* (1–62.5).

We originally set out to isolate mutants affected in memory (in contrast to those blocked in immediate learning) because memory mutations should act in the central nervous system. The fact that *amnesiac* learns in the first place indicates that its sensory and motor systems are functioning properly. Moreover, the action of memory mutations should be relatively well defined. To learn, a fly must sense cues and reinforcement, transmit the information to the proper neural centre, encode the association, and later convert it to appropriate motor output. This is a series of complex processes; a mutation could affect any of them. In contrast, memory retention requires two things—that information be stored in a relatively permanent form and that it be accessible to retrieval. We believe the *amnesiac* mutation affects one of these, probably accessibility to retrieval. Further understanding of the mutation's action on memory will depend on finding anatomical or biochemical abnormalities in *amnesiac* flies.

Gaining substantive information about memory retention will probably require more than one mutant. It is worth noting that other mutations can affect memory. Y. Dudai (personal communication) has evidence that *dunce* flies³ store learned information in a labile form for a very short time after training. We have isolated a mutant, *turnip*(*tu*^{PS274}) which is apparently blocked in immediate learning. The heterozygote, *turnip*/+ learns normally but forgets very rapidly (Fig. 3). A plausible explanation for this situation is that the homozygote, more severely affected in retention, forgets immediately after training so that no learning can be detected.

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Interactions between *Entamoeba histolytica* and complement

THE pathogenic mechanisms in amoebiasis are not fully understood. Histopathological studies of infected tissues have shown lysis of host cells near the parasites, particularly in the liver where abscesses are formed. In the intestine, inflammatory reactions are often slight, unless there is secondary bacterial involvement. The infiltrating cells belong mainly to the lymphocyte and macrophage cell series, and neutrophil leukocytes are sparse¹, suggesting that there is a low-grade chronic inflammation. Tacheuchi and Phillips² have made systematic studies of experimentally infected germ-free guinea pigs, which have revealed the early events in invasive amoebiasis. When the amoebae had not yet made contact with the colonic epithelial cells, the latter showed degenerative changes such as loss of brush borders, swelling of mitochondria and dilatation of the endoplasmic reticulum. At the same time polymorphonuclear leukocytes (PMN) as well as some macrophages were seen to infiltrate the lamina propria, and extravasated erythrocytes were observed in the same areas. A high proportion of the PMN showed signs of degeneration, including disruption of the cell membrane. As changes were observed some distance from the parasites, Tacheuchi and Phillips² concluded that the parasites exert their effects by means of a soluble mediator(s), possibly a toxin elaborated by the parasites. Another possibility is that enzymes released from amoebae damage both epithelium and PMN. A third explanation would be that the amoebae can interact with host material to generate factors which are chemotactic for leukocytes and can also exert toxic effects. A likely candidate would be the complement system. For this reason, the experiments described here were undertaken to analyse interactions between amoebae and the complement system. IgG antibody fractions were prepared from sera of individuals suffering from amoebiasis, and unheated sera from normal humans or experimental animals were used as a source of heat-labile complement components. We found that *Entamoeba histolytica* activates complement by the alternative pathway, and can be lysed by the reaction products.

Table 1 % Of amoebae lysed by human sera in various experimental conditions

Serum	Unheated	Heated	Unheated +EDTA	Unheated +EGTA	Unheated -properdin
BF	37	2	2	43	5
GH	46	2	5	50	2
BH	42	3	1	51	NT
IC	39	1	3	44	NT
PH	43	2	6	46	NT
Normal rabbit	NT	3	NT	NT	NT

Hk 9 strain of *E. histolytica*, cultivated axenically, Normal human sera, lacking demonstrable antibodies, were diluted 1:3 without heating or after heating at 56 °C for 30 min; EDTA or EGTA were used in final concentration of 5 mM. Assays were carried out in cytotoxicity trays (Møller-Coates), using 6 µl containing about 200 amoebae. Assays were carried out in quadruplicate using 0.1% trypan blue, counting a total number of 400–500 cells. Sera were depleted of properdin by affinity chromatography on an anti-properdin-activated Sepharose column at 4 °C in the presence of 10 mM EDTA (ref. 14) which was later removed by dialysis in the cold. After this procedure the serum was still capable of lysing amoebae in the presence of antibodies.

Two strains of *E. histolytica*, Hk 9 and NIH 200, were grown in culture. When amoebae from either one of these strains was incubated in fresh human serum without detectable antibodies, a high proportion of the amoebae were lysed, as shown by marked morphological changes, including disruption of the cell membrane and expulsion of nuclei. These results were

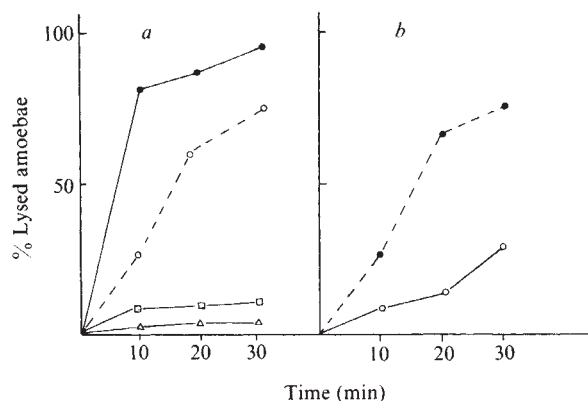


Fig. 1 % Dead amoebae (Hk 9) after incubation for 10, 20 and 30 min with unheated or heated normal human serum diluted 1:2 in phosphate-buffered saline, pH 7.4, containing Ca^{2+} and Mg^{2+} (PBS). *a*, ●, Amoebae preincubated for 30 min at 4 °C with the IgG fraction of a human serum containing antibodies to *E. histolytica*. The fraction was obtained by ion-exchange chromatography on a DEAE Sephadex A50 column. Immunofluorescence with class-specific antiglobulin conjugates showed an IgG titre of 40, IgM and IgA negative. After interaction with IgG antibodies, fresh unheated human serum with no detectable antibodies was added to give a final dilution of 1:2. ○, Amoebae preincubated at 4 °C for 30 min in PBS followed by reaction with unheated serum. □, Preincubation with IgG antibodies followed by heated serum. △, Preincubation in PBS followed by heated serum. *b*, ○, Preincubation with IgG antibodies followed by unheated serum + 5 mM EGTA. ●, Preincubation without antibodies followed by unheated serum + 5 mM EGTA.

confirmed by the release from prelabelled cells into the medium of either ^3H -uridine or ^{14}C - α -isobutyric acid. However, the use of isotopes was somewhat less sensitive, as well as less convenient, than morphological observations, and for most experiments the latter were used. The lysis was inhibited by 5 mM EDTA, which chelates both Ca^{2+} and Mg^{2+} ions, but not by 5 mM EGTA, which selectively chelates Ca^{2+} ions. When sera were depleted of properdin, lysis did not occur (Table 1). Lysis was efficient in the presence of $\geq 50\%$ normal guinea pig serum as well as C4-deficient guinea pig serum, in which there is a complete block of the classical pathway (Table 2).

Two distinct effects of antibodies were demonstrated. If amoebae were incubated with the IgG fraction of an antiserum and then with unheated serum, accelerated lysis was observed, presumably due to classical pathway activation. However, the lysis occurring in the presence of EGTA was markedly inhibited (Fig. 1). This may be due to steric hindrance by antibodies of access to groups on the amoeba surface with alternative-pathway activating properties.

Immunofluorescence showed the presence of C3 or a derivative with the same immunological reactivity on the surface of the amoebae. Capping followed by endocytosis occurred in a similar way to that described by Aust Kettis and Sundqvist³ in their studies of the capping of specific antibodies on the surface of *E. histolytica*.

These observations demonstrate that *E. histolytica* is able to activate complement by the alternative pathway and that the parasites can be lysed by the reaction products. Furthermore, sera from rabbits congenitally deficient in C6 gave markedly reduced lysis compared to sera from normal rabbits, and depletion of normal human serum of C5 by affinity chromatography abolished its capacity to lyse amoebae. These findings indicate that lysis is due to the terminal complement components (C5–C9).

Serum dilutions higher than 1:10 resulted in very little lysis. Experiments were designed to ascertain whether such sub-lytic concentrations of complement might stimulate amoebae to increased secretion of hydrolytic enzymes in a manner analogous to the secretion-stimulating effects of immune complexes⁴

and isolated C3b (ref. 5) on macrophages. In repeated experiments it was found that both the total concentration and release of the acid hydrolase *N*-acetyl- β -D-glucosaminidase (NAGase) were increased in amoebae which were grown in the presence of 5–10% unheated serum, in contrast to comparable amounts of heated serum (Fig. 2).

The relevance of these observations to the pathogenesis of amoebiasis is not yet known. Activated complement may participate in defence against amoebae. Nevertheless, despite the lytic effect of complement, *E. histolytica* can in many instances survive in the tissues. Continuous turnover of parasite cell membrane components accompanied by change in distribution of complement receptors may contribute to their survival in the host. Moreover, in the intestinal wall the concentration of active complement components may be at the limit of what is required to lyse the parasite. In this situation complement cleavage products, such as C3b, might stimulate the amoebae to increased production and release of hydrolytic enzymes. Such enzymes

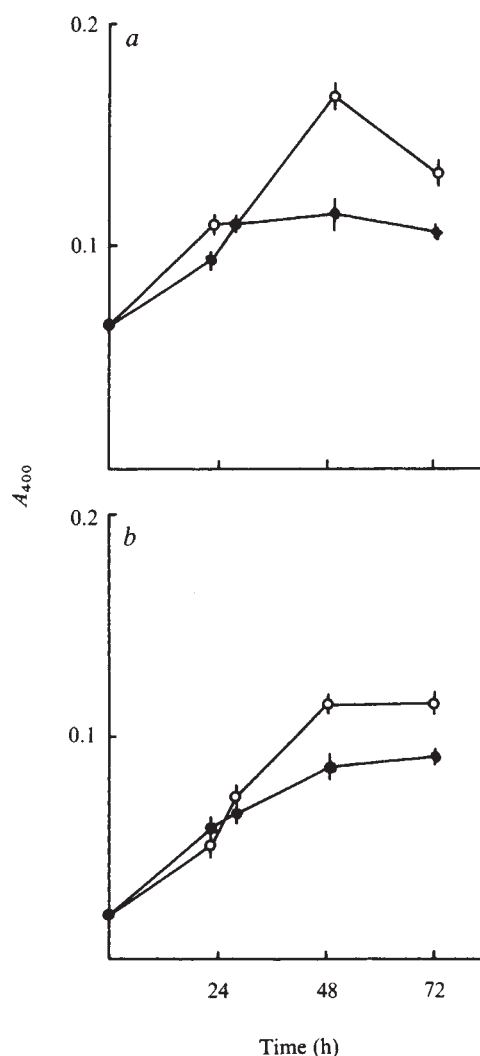


Fig. 2 *a*, Total amount of NAGase per 1000 amoebae; *b*, amount of enzyme released into the medium per 1000 amoebae. *E. histolytica* (NIH 200) was cultured in 1.8-ml glass tubes with screw caps (PBG-Trident) in medium TPS-1 with the addition of either 10% heat-inactivated or 5% heat-inactivated and 5% unheated human serum. At 22, 28, 49 and 72 h four tubes in each series were chilled in an ice bath, shaken in a whirl mixer and 1 ml removed for parasite counting in a Coulter Counter Industrial B. The remainder were centrifuged for 5 min at 1,500 r.p.m. Enzyme assay according to Woollen *et al.*¹⁵ was carried out on supernatants and cells. ●, 10% heated human serum; ○, 5% heated + unheated normal serum.

seem to be involved in the pathogenesis of many chronic inflammatory conditions⁶, and the combined effects of activated complement, leukocyte products and amoebic enzymes probably contribute to the lytic and reactive changes observed in the tissues of patients with invasive amoebiasis.

Table 2 % Of amoeba lysed by normal and C4-deficient unheated and heated guinea pig serum in various experimental conditions

Serum	% Dead parasite cells
Unheated	57
Unheated + EDTA	2
Unheated + EGTA	75
Heated	5
C4 deficient, unheated	54
C4 deficient, heated	3

E. histolytica strain NIH 200 was incubated with 3:4 dilutions of guinea pig sera. Other experimental conditions as for Table 1.

Capacity to activate complement by the alternative pathway has also been reported for other parasites, including *Trypanosoma cruzi*^{7,8}, African trypanosomes, including the variant-specific antigen⁹⁻¹¹, and *Schistosoma mansoni*¹². Schistosomula incubated with normal serum activate complement by the alternative pathway, and the C3 on the surface of the parasites allows attachment of eosinophils and killing of the parasites¹³. Hence, activation of complement by the alternative pathway can not only lyse parasites directly, as described here for enteropathic amoebae, but also render parasites susceptible to complement-dependent cell-mediated lysis. Complement activation, presumably initiated by surface glycoproteins of parasites, may be a relatively common phenomenon, contributing on the one hand to host protection and on the other hand to the pathogenesis of parasitic diseases.

Sera from rabbits congenitally deficient in C6 were obtained from Professor K. Rother, goat anti-human C5 serum was provided by Dr D. R. Schultz, strains of *E. histolytica* by Dr L. Diamond, absorbed rabbit anti-properdin serum by Dr C. O. Kindmark, and C4-deficient guinea pig serum by Dr P. Lachmann.

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HLA and testicular cancer

HLA is involved not only in transplantation, but is also associated with many disease states. This first became evident when the major histocompatibility complex of the mouse (H-2) was shown to play a major part in determining resistance to Gross leukaemia virus¹. Subsequent studies in both animals and man have shown that many disease states are associated with, and perhaps regulated by, the major histocompatibility complex^{2,3}. We now present the tissue typing results from a group of patients with testicular cancer. The results show an abnormally high antigen frequency for HLA-Dw7 in patients with testicular teratocarcinoma.

The study population consisted of 61 patients from the University of Minnesota. Thirty patients had seminoma, 27 had embryonal cell carcinoma, 26 had teratocarcinoma, and 7 patients had choriocarcinoma (some of the patients had mixed tumours). All patients had completed treatment (surgery, radiotherapy, and/or chemotherapy) and were clinically free of disease at the time of testing. Peripheral blood lymphocytes were obtained on each individual by Ficoll-Hypaque density centrifugation⁴ and frozen at a controlled rate in 10% dimethyl sulphoxide and RPMI 1640 with 25 mM HEPES (Microbiological Associates) supplemented with 20% pooled human serum (PHS), heparin (20 IU ml⁻¹), L-glutamine (0.2 mg ml⁻¹), streptomycin (100 µg ml⁻¹) and penicillin (100 IU ml⁻¹). The cells were stored in vapour phase liquid nitrogen until ready for analysis at which time they were thawed rapidly at 37 °C and diluted with 5% PHS, washed twice, and resuspended in culture media for testing. All cells were tested for 52 HLA-A, HLA-B and HLA-C specificities by the NIH standard technique⁵. HLA-D assignments were made by homozygous typing using at least 22 typing cells covering all HLA-D specificities except HLA-Dw8 and HLA-Dw9. Homozygous typing was performed in round-bottomed microtitre plates using 5 × 10⁴ responders and 5 × 10⁴ stimulator typing cells inactivated with 5,000 rad from a ¹³⁷Cs source (3.2 min). Cells were suspended in 0.2 ml of 20% PHS and incubated in a 37 °C, 5% CO₂ and air atmosphere for 6 days. They were then terminated immediately after a 6-h pulse with 1.0 µCi ³H-TdR (6.7 Ci mmol⁻¹, NEN). Cells were collected on an automated harvester. Results were 'double-normalised' and typing responses were assigned to those cells that gave less than 35% DNV. The reactivity of patient cells was checked by stimulation with purified PHA (HA-17) (Wellcome), and only those patients responding in the same range as normal control cells (typed simultaneously with the tumour patients) were included for the HLA-D typing results. Antigen frequencies were then calculated for the tumour group as a whole as well as for each of the groups of patients with seminoma, embryonal cell carcinoma and teratocarcinoma, and compared to normal control values (*n* = 561 for HLA-A and HLA-B, 171 for HLA-C and 150 for HLA-D specificities) obtained from a random population of the same geographical area as the tumour patients. Significance was determined by χ^2 analysis and *P* values were corrected to account for chance significance; *P* < 0.01 was considered significant⁶.

The antigen frequency of HLA-Dw7 was significantly increased in the teratocarcinoma group (12/26, *P* < 0.01) as compared to normal control values (14/150) (Table 1); the difference, however, was not detected in either of the seminoma (7/24, not significant), choriocarcinoma (0/7, NS) or embryonal cell carcinoma groups (7/24, NS) or the total patient group (15/53, NS). (Six patients were eliminated from the original seminoma group (30) and 3 patients from the original embryonal carcinoma group (27) for HLA-D typing because of inadequate reactivity to HA-17.) The relative risk (which is a calculated term that represents the risk of developing a disease in an individual possessing a particular HLA antigen as

Table 1 Frequency of HLA-D determinants in patients with testicular teratocarcinoma and controls

Determinant	Normal controls (%)	Patients with teratocarcinoma (%)	P value
Dw1	19/150 (13)	7/26 (27)	NS
Dw2	19/150 (13)	3/26 (12)	NS
Dw3	21/150 (14)	6/26 (23)	NS
Dw4	12/150 (8)	2/26 (8)	NS
Dw5	7/100 (7)	1/26 (4)	NS
Dw6	0/50 (0)	1/26 (4)	NS
Dw7	14/150 (9)	12/26 (46)	<0.01
Dw10	5/150 (3)	0/26 (0)	NS
Dw11	18/150 (12)	0/26 (0)	NS
Single blank	69/168 (41)	8/26 (31)	NS
Double blank	30/168 (18)	6/26 (23)	NS

HLA-D typing was performed as described in the text. Lymphocyte responsiveness for each patient was checked by response to purified PHA (HA-17). 5×10^4 cells were incubated in 0.2 ml of 1:100 HA-17. Cultures were terminated at 96 h immediately after a 6-h pulse with $1.0 \mu\text{Ci } ^3\text{H-TdR}$ (6.7 Ci mmol^{-1}). Only those cells responding in the range of normal controls were included in the HLA-D typing results. All HLA-Dw7 assignments were made by typing responses to HTC JB-12 (submitted to the Seventh International Histocompatibility Workshop, Oxford, as 7w542) and HTC 56 (submitted as 7w534). One teratocarcinoma patient was assigned HLA-Dw1, Dw6, Dw7 and included in the HLA-Dw7 group; however, if this patient were excluded from analysis, the increased HLA-Dw7 antigen frequency would still be significant (11/25, $P < 0.01$). NS, not significant.

compared to an individual lacking that antigen) between HLA-Dw7 and teratocarcinoma was 8.32. The association between HLA-Dw7 and teratocarcinoma, although statistically significant, should be interpreted with the understanding that the HLA-D assignments are based on reactivity of responder (tumour patients') T cells which can be affected by the presence of cancer as well as its various modes of treatment. In order to reduce this source of error, lymphocyte reactivity from each study patient was checked with purified PHA (HA-17) and only those responding in the same range as normal control cells were included in the HLA-D typing results.

The biological significance of this as well as other disease associations with HLA is, as yet, unknown. However, four possible mechanisms have been suggested. (1) HLA antigens may cross-react with pathogen antigens leading to cross-tolerance; the pathogen, then would not be subjected to the host's immune mechanisms. (2) HLA antigens may function as cell surface receptors for particular pathogens similar to the association between malaria susceptibility and Duffy red blood cell antigens⁷. (3) There may be a genetic linkage between genes controlling the expression of HLA antigens (which serve as markers) and genes controlling immune responsiveness of the host and likewise, (4) HLA antigens may serve as markers for specific genes causing deficiency of specific normal proteins similar to the HLA linked genes regulating levels of second and fourth components of complement⁸.

Irrespective of which of these mechanisms apply, the association between HLA and testicular teratocarcinoma is especially interesting for two reasons—first, it has been suggested that heredity may be an aetiological factor in the development of testicular cancer because at least 18 cases of familial testicular cancer have been reported⁹. And second, there is a relationship between teratocarcinoma and H-2 in the mouse¹⁰; specifically, the H-2 linked T/t complex which exerts control over embryonic differentiation, male reproduction including sterility and transmission ratio distortion, and crossing over frequency between T/t and H-2 (refs 11, 12) also controls the expression of an embryonic antigen, F9, that is expressed on adult teratocarcinoma cells^{13,14}. In addition, the F9 antigen is similar to H-2 antigen in molecular weight and subunit structure and may, in

fact, represent an embryonic equivalent of histocompatibility antigens¹⁵.

The association between the human major histocompatibility complex (HLA) and testicular teratocarcinoma is therefore of interest considering the homology between mouse and the human major histocompatibility complex. First, because F9 and H-2 K and D region antigens have closely associated molecular and subunit structures¹⁵, a similar teratocarcinoma associated antigen in the human may cross-react with HLA (HLA-Dw7) leading to a state of 'cross-tolerance' which would support one of the theories of HLA and disease association. Second, considering the established relationship between the major histocompatibility complex, the T/t complex, and teratocarcinoma in the mouse system, a relationship between HLA-Dw7 and teratocarcinoma may add support to the existence of a MHC linked 'T/t like' region in the human. This concept is further supported by the association between HLA and spina bifida which may be considered to have morphological similarities to those controlled by the T/t complex. In addition, there are antigens cross reacting with F9 on human sperm¹⁷ as well as on human teratocarcinoma¹⁸.

Thus, in the mouse there is a structural and developmental association between a teratocarcinoma associated antigen (F9) and the major histocompatibility complex. The association of HLA-Dw7 with testicular teratocarcinoma could lead to similar studies in the human that will further explain the function of the human major histocompatibility complex.

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Specific antibody-secreting cells generated from cells producing immunoglobulins without detected antibody function

STUDIES of immune responses in rabbits, rats and mice have led us to describe a population of differentiated immunocytes synthesising and secreting immunoglobulins without detectable antibody function for the antigens injected¹ (IFC). These cells were antigen induced and appeared before the antibody-forming cells (AFC). The proliferation of IFC after antigenic stimulation can be explained by several hypotheses, the most plausible being that antigen-stimulated T cells release nonspecific factors which trigger clones of B cells bearing membrane receptors unrelated to the inoculated antigen. Such an explanation has been advanced by several authors^{2,3} but experiments performed in our laboratory do not support such polyclonal activation. Indeed, some cells contained both antibodies and immunoglobulins without detectable antibody function in different areas of their cytoplasm^{1,4}. Furthermore, some common idiotype specificities were expressed by both AFC and IFC (ref. 5). Based on these findings, and additional studies on the developmental kinetics of IFC and AFC in animals with different immunological status, which indicate that there is a close relationship between these two cell populations, we put forward the hypothesis that AFC might be recruited among IFC. That is, that these two cell types represent two stages of differentiation of the same cell population. To test this assumption directly we performed experiments at the single cell level with micromanipulated and individually cultured IFC and found that at least some of these cells generate specific AFC.

Adult C57BL/6 strain mice were immunised by injection of horseradish peroxidase (PO) emulsified in Freund's complete adjuvant into both hind footpads. Six days later, cell suspensions were prepared from their popliteal lymph nodes and tested for the presence of AFC and IFC. Anti-peroxidase antibody-secreting cells (AFC) and immunoglobulin-secreting cells were detected by Cunningham's⁶ and reverse^{7,8} local haemolysis plaque assays respectively (see legend to Table 1). At this time,

Table 1 Number of IFC, anti-peroxidase and anti-SRBC AFC per 10^6 viable lymph node cells after 24 h of mass culture*

IFC	Anti-peroxidase AFC		Anti-SRBC AFC	
	Direct plaques	Indirect plaques	Direct plaques	Indirect plaques
872 ± 474	1 ± 4	4 ± 6	1 ± 2	1 ± 3

C57BL/6 mice were injected in both hind footpads with 250 µg of horseradish peroxidase (PO) emulsified in Freund's complete adjuvant and killed 6 d later. Popliteal lymph nodes were removed, teased and cells were mass cultured in conditions reported in detail previously⁹⁻¹¹. Briefly, 8×10^6 viable lymphoid cells were cultured without shaking in 35 mm Petri dishes containing 2 ml Click's medium¹² supplemented with LPS (*Escherichia coli* 055B5 lipopolysaccharide, Difco) at a final concentration of 4 µg ml⁻¹ and with PO covalently linked to horse red blood cells¹³ (HRBC-PO, 5×10^6 per dish). The cells were collected 24 h later and the anti-PO, anti-sheep red blood cell (SRBC) AFC and immunoglobulin-secreting cells were detected by local haemolysis plaque assay, the indicators used being either SRBC covalently coupled to PO (ref. 13), SRBC or SRBC coated with rabbit hybrid antibody (Fab')₂ fragments with dual specificity to SRBC and mouse immunoglobulins (refs 7, 8). SRBC adsorbed guinea pig serum was used as source of complement. To reveal the indirect AFC and immunoglobulin-secreting cells, a rabbit anti-mouse Ig antiserum was added to the mixture (final dilution used: 1/400).

* Arithmetic means of 19–24 different experiments ± one standard deviation. In each experiment the lymph nodes of 5–12 mice were pooled.

Table 2 Percentage of IFC which generate anti-PO AFC or which change into anti-PO AFC

No. of micromanipulated IFC	Presence(+) or absence(-) of peroxidase inhibitor	No. and percentage (in parentheses) of wells containing AFC after 48 h of microculture	Total no. of haemolytic plaques detected with SRBC-PO after 48 h of microculture
392	–	90 (23)	345
119	+	2 (1.7)	3

Lymphoid cells were collected after 24 h of mass culture (see legend to Table 1). Revealed IFC were micromanipulated from the centre of haemolytic plaques by a previously described procedure^{10,11} and transferred into separate wells of polyacrylamide plates previously seeded with 10^3 to 10^4 irradiated (1,500 rad) normal C57BL/6 mouse splenocytes or thymocytes as feeder layer and 10^3 to 5×10^3 HRBC-PO as antigen. The medium used for the microculture was composed of 50% fresh Click's medium and 50% previously filtered supernatant of the mass culture. After 48 h of microculture, the content of each well was removed and mixed with SRBC-PO and complement. Cells lysing these red cells were detected by Cunningham's haemolysis plaque assay. The specificity of the molecules secreted by the progeny of microcultured cells capable of lysing SRBC-PO was tested in control experiments by adding soluble peroxidase as inhibitor to the revealing mixture (final concentration 70 µg ml⁻¹).

801 ± 301 IFC per 10^6 viable lymph node cells were present, but no AFC were found. The lymphoid cells were mass cultured for 24 h to select the most resistant cells under *in vitro* conditions. After this culture, the number of IFC did not vary and very few anti-PO and anti-sheep red blood cell (SRBC) AFC appeared (Table 1). Some of these IFC were micromanipulated, transferred into separate wells of polyacrylamide rafts^{14,15} and individually cultured in the conditions described in Table 2, in the presence of irradiated syngeneic splenocytes or thymocytes as feeder layer and horse red blood cells covalently linked with PO (HRBC-PO) as stimulating antigen. The scarcity of indirect anti-PO and anti-SRBC AFC detected at the time of micromanipulation made it highly improbable that such PFC would be micromanipulated (Table 1). Almost all the micromanipulated cells can therefore be considered as cells really secreting immunoglobulins without detectable antibody function.

After 48 h of culture, 90 out of 392 micromanipulated IFC (23%) were transformed (in 34% of positive wells only one haemolytic plaque was detected) into cells able to lyse SRBC-PO or generated daughter PFC lysing these erythrocytes. Only some of the individually cultured cells yielded a PFC progeny. In

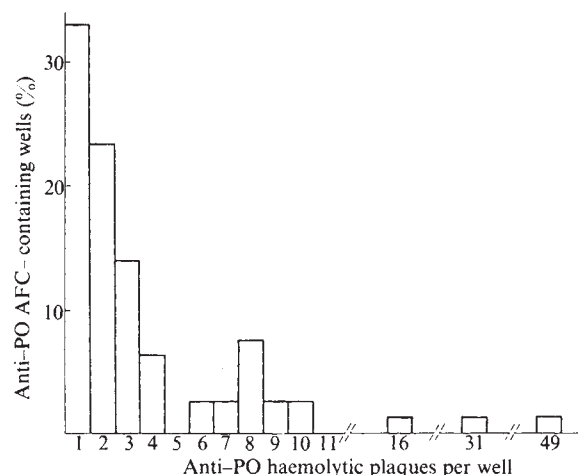


Fig. 1 Distribution of the number of anti-PO haemolytic plaques found in each positive well seeded 48 h before with a single micromanipulated IFC.

previous experiments of this type, about 10–20% of parental PFC generated daughter PFC (refs 11, 15) the others could be either terminal cells, cells generating memory cells or cells injured by micromanipulation procedure. It has been shown¹¹ that PFC are derived from micromanipulated cells and not from the irradiated cells used as the feeder layer, and this was further tested in the present study (unpublished results). Thus, no anti-PO AFC was found in wells containing only irradiated splenocytes or thymocytes and HRBC-PO, irradiated splenocytes or thymocytes used as the feeder layer were equally effective for the generation of AFC from IFC, and if the number of irradiated splenocytes was varied from 10^3 to 10^4 cells per well, the same percentage of wells containing anti-PO AFC was found.

Of the wells containing anti-PO AFC, 66% had more than one haemolytic plaque (see Fig. 1). Often, the centre of large haemolytic plaques contained several clustered cells. It is therefore likely that one or more divisions of the micromanipulated cells were needed before the secretion of anti-PO antibodies by the daughter cells could be detected.

Table 3 Some AFC derive from IFC. This event requires the presence of the immunising antigen

No. of micromanipulated IFC	Presence or absence of PO in the microculture	No. of wells containing AFC after 48 h of microculture	Total no. of haemolytic plaques detected on SRBC-PO after 48 h of microculture
110	HRBC-PO	30 (27.3%)	174
127	HRBC	2 (1.6%)	8

Micromanipulated IFC were transferred into separate wells of polyacrylamide rafts containing 5×10^3 irradiated (1,500 rad) normal C57BL/6 mouse splenocytes and either 2.5×10^3 HRBC-PO, or 2.5×10^3 native HRBC. For conditions of the microculture and detection of daughter PFC see Table 2.

The specificity of the antibody secreted by the daughter cells lysing the SRBC-PO was checked by adding soluble peroxidase in the revealing mixture. In these control experiments, only 2 out of 119 wells tested contained 3 haemolytic plaques, (compared to 90 out of 392) (Table 2). The 97% inhibition of haemolytic plaques when soluble peroxidase was present in the revealing mixture and the absence of antigenic determinants shared by SRBC and PO (ref. 8 and unpublished results) demonstrate that cells lysing the SRBC-PO were true anti-PO antibody producers.

To determine whether the presence of the immunising antigen was critical to the generation of AFC from IFC, some of the latter cells were micromanipulated and transferred to wells containing irradiated cells and native HRBC (Table 3). Control experiments where IFC were microcultured into wells containing HRBC-PO, were run simultaneously. Only the IFC cultured in the presence of HRBC-PO generated a large number of anti-PO AFC. In contrast, very few anti-PO haemolytic plaques were detected in wells seeded with native HRBC (25 times less).

The experiments reported in the present paper clearly demonstrate that some IFC taken from mice immunised with PO generate anti-PO AFC or change into anti-PO AFC. This event seems to be associated with one or several cell divisions and requires the presence of the antigen. It appears, therefore, that IFC and AFC do represent different stages of differentiation of the same cell population. One explanation is that IFC and precursors of IFC are monopotent cells, that is, there is only one antibody-forming potentiality per cell, the IFC synthesising and secreting non-functional precursors of antibodies. Such an explanation is not substantiated by the biochemical study we have performed on these cells¹⁶. Indeed it has not been possible to detect any particular composition or molecular weight for the immunoglobulins synthesised by the IFC. Furthermore the finding that the presence of the antigen is required for this step of

differentiation to occur makes this type of explanation improbable. Alternatively IFC and precursors of IFC may be multipotent cells, that is, they synthesise and secrete immunoglobulins with multiple specificities. During differentiation, and under the selective influence of the antigen, they acquire a specialisation, that is, they synthesise monospecific antibody molecules. The finding that the presence of the antigen is required for this step of differentiation to occur lends further weight to such a view. This event may require genetic rearrangement, occurring during cell division.

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Murine embryonic B lymphocyte development in the placenta

B LYMPHOCYTES derive from pluripotent stem cells which give rise to erythrocytes, granulocytes, monocytes, macrophages and T and B lymphocytes^{1,2}. Stem cells have been found in yolk sac, fetal liver, bone marrow, spleen and blood. During ontogeny from stem cells to mature B cells, precursor B cells (pre-B cells) can be found. Various types of pre-B cell can be identified. There are those which already produce and release immunoglobulin (Ig), but which are morphologically different from mature B cells^{3,4}. Using surface-bound Ig as a marker for the development of mature B cells, it has previously been found that fetal liver, but not yolk sac, could develop mature B cells *in vitro*^{5,6}. A second type of pre-B cell can be characterised functionally as not being reactive to antigen or mitogens but which, on adoptive transfer into irradiated recipients or following *in vitro* culture, develops into a reactive cell^{7–9}. When stimulated by antigens or mitogens, such mature, reactive surface Ig-bearing resting B lymphocytes develop into IgM-, IgG-

Table 1 H-2 haplotype of LPS-induced PFC*

Placenta (embryo)	Treatment	Complement only	Anti-DBA/2J (H-2 ^b anti-H-2 ^d)	Anti-C57BL/6J (H-2 ^d anti-H-2 ^b)
(C57BL/6J × DBA/2J)F ₁ (H-2 ^b × H-2 ^d)	(Reduction)	500 ± 100	60 ± 30 (88%)	50 ± 40 (90%)
Spleen (adult, mother) C57BL/6J (H-2 ^b)	(Reduction)	850 ± 150	820 ± 200 (4%)	45 ± 20 (95%)
Spleen (adult, father) DBA/2J (H-2 ^d)	(Reduction)	550 ± 100	20 ± 15 (97%)	480 ± 100 (12%)
Spleen (adult, F1) (C57BL/6J × DBA/2J)F ₁ (H-2 ^b × H-2 ^d)	(Reduction)	920 ± 120	90 ± 30 (90%)	40 ± 20 (95%)

* Duplicate aliquots of the IgM-secreting PFC at the peak of the response were first incubated with C57BL/6J (anti-DBA/2J spleen cell) serum (H-2^b anti-H-2^d) or with DBA/2J (anti-C57BL/6J spleen cell) serum (H-2^d anti-H-2^b) (both gifts from Dr H. von Boehmer) for 30 min on ice, thereafter washed and then incubated with appropriately diluted rabbit complement for 30 min at 37 °C. Finally, the number of IgM-secreting PFC was determined in the protein A-SRC plaque assay¹¹. PFC were treated this way for placenta cell cultures (at day 12 of gestation) at time equivalent to the time of birth (day 19 of gestation) in culture, and at day 5 of culture for spleen cells grown from 3×10^5 cells ml⁻¹ in the presence of LPS.

and IgA-secreting plasma cells, which can be detected using a haemolytic plaque assay as plaque-forming cells (PFC)^{10,11}. Pre-B cells which develop into antigen- or mitogen-reactive cells have been localised in fetal liver, bone marrow and spleen. The earliest that pre-B cells had previously been identified during embryogenesis of the mouse was at about day 12 or 13, but I report here the detection of IgM- and IgG-secreting PFC in mitogen-stimulated cultures of single cell suspensions of mouse placenta 9–14 d old. Placenta is thus identified as a new site of B-lymphocyte development which, during embryogenesis, precedes B-cell generation in fetal liver.

The culture technique used facilitates *in vitro* development of pre-B cells from the placenta to mature B-cells which can subsequently be stimulated by the B-cell mitogen lipopolysaccharide (LPS) to IgM- and IgG-secreting cells. Placentae were obtained from timed pregnant C57BL/6J female (H-2^b) mice, mated with DBA/2J male (H-2^d) mice. The embryo was first

removed from the uterine wall and then the ectoplacental cone was dissected away from the uterus, and thereafter, from the yolk sac and embryo. They were rinsed extensively with balanced salt solution (BSS) and then dispersed into single cell suspensions by cutting the cones into small pieces with scissors, followed by passaging of the placental pieces through a stainless screen (200 mesh) with the aid of the piston of a 5-ml plastic syringe. After washing three times with cold BSS, the placental cells were suspended at 5×10^6 cells ml⁻¹ in medium containing LPS. Culture conditions were those described previously for fetal liver cells⁸, except that dextran sulphate was added. The addition of dextran sulphate increased the total LPS-induced IgM-PFC response of placental cells five- to tenfold (details to be published elsewhere), but the development of IgM- and IgG-PFC in cultures of placental cells was still dependent on the presence of the B-cell mitogen LPS.

Cells obtained from placenta at any time during gestation (between days 9 and 14) yielded a peak of IgM-secreting PFC in culture at the time corresponding to day 19 of gestation (Fig. 1). This was followed by the peak of IgG-secreting PFC 2–5 d later (not shown). As shown previously⁸, the largest number of IgM-secreting PFC developing from mature B-cells is usually observed in such cultures 4–5 d after mitogenic stimulation. If it is assumed that maturation of B cells from placenta *in vitro* parallels in time their maturation *in vivo*, then placental cells, kept either *in vivo* or *in vitro*, acquire LPS reactivity probably at the equivalent of days 14–15 of gestation. The highest number of precursor B cells was observed in placental cells removed on day 12 of gestation, as estimated by the total number of PFC obtained in the mitogenic response (Fig. 1).

The majority of all IgM-secreting PFC developing in placental cell cultures expressed the embryonic H-2^b and H-2^d haplotypes (Table 1) following LPS stimulation. This excludes the possibility that the LPS-reactive cells were contaminating maternal (H-2^b) cells.

The frequency with which precursor cells in the placenta developed LPS reactivity and then into IgM-secreting PFC in culture, was determined by limiting dilution analyses in the presence of rat thymus 'filler' cells (3×10^6 per ml) (ref. 9). Placental cells at day 10 of gestation contained one precursor in 2×10^4 cells; at day 11, one in 4×10^3 ; at day 12, one in 8×10^2 ; at day 13, one in 5×10^3 ; and at day 14, one in 10^5 cells. Placenta, therefore, precedes fetal liver as an organ of embryonic B-cell development by several days⁹. As placental B-cell development declines, it rises in fetal liver (Fig. 2).

B-lymphocyte development in the mammalian embryo has been recognised to be multifocal. Furthermore, it is now evident that the sites of B-lymphocyte development change with the stage of embryogenesis. A first wave of B-cell differentiation in

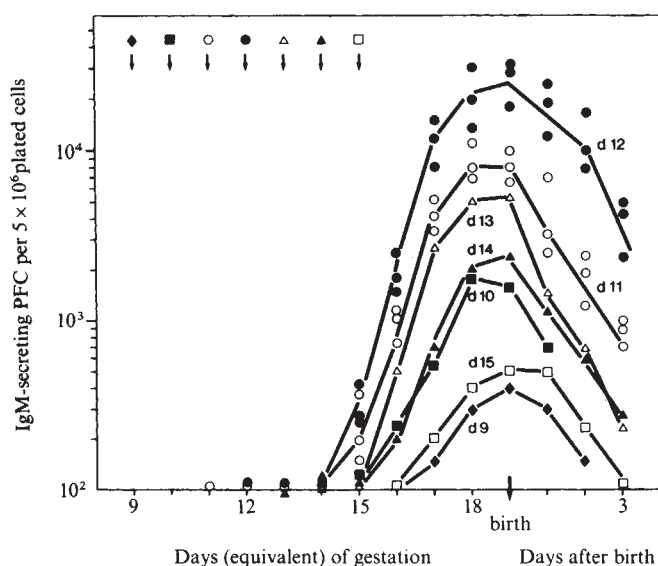


Fig. 1 The development of IgM-secreting PFC (determined with the protein A-SRC assay¹¹) in suspension cultures of placenta at different stages of gestation (◆, day 9; ■, day 10; ○, day 11 (3 experiments); ●, day 12 (3 experiments); △, day 13; ▲, day 14) in the presence of dextran sulphate ($100 \mu\text{g ml}^{-1}$; MW 500,000; Serva) and LPS ($50 \mu\text{g ml}^{-1}$; a gift from Drs C. Galanos and O. Lüderitz, Max-Planck Institut für Immunbiologie, Freiburg, West Germany) assayed at various days during culture.

the placenta up to day 12 of gestation is followed by a second wave in fetal liver, which continues to birth. Thereafter, spleen and bone marrow take over as major sites of B-cell development. Placental B-cell generation begins concomitantly with the development of blood islands in yolk sac and ceases (at day 12) when haematopoiesis ceases in the yolk sac. Placenta and yolk sac are in intimate association during this time of embryogenesis¹². It is, therefore, possible that pluripotent stem cells or B-cell precursors migrate from yolk sac to placenta for further differentiation along the B-cell pathway.

Yolk sac is known to contain pluripotent stem cells from which mature erythrocytes, granulocytes, monocytes, megakaryocytes and lymphocytes arise by differentiation. Cell-marker experiments have shown that stem cells migrate, and are thereby seeded into embryonic myeloid organs and primary lymphoid organs, such as fetal liver, bone marrow, spleen and thymus¹³. Stem cells themselves are thought to be uncommitted; it has been suggested that their differentiation into the various types of blood cells depends on the inductive influence of the organ rudiment into which they migrate¹⁴. In avian blood cell differentiation, it is possible that the epithelium of the bursa of Fabricius could have such an inductive effect, although there is no indication as to the genetic loci which may control the expression of the inducing signals on such epithelial cells. Neither is anything known of the possible genetic control of murine B-cell development through genes controlling the expression of B-cell differentiation-inducing signals. If such differentiation-inducing structures exist, they are probably expressed on mesenchymal-endodermal elements¹⁵ of placenta, fetal liver, bone marrow and spleen. B-cell differentiation in the placenta, however, may offer embryonic stem cells the possibility to differentiate in response to signals which are encoded in the maternal genes, expressed on maternal endoderm within the placenta.

The site of B-cell differentiation certainly does not depend on the site of stem-cell generation. Stem cells could migrate to the placenta either from yolk sac¹³ or from another embryonic site¹⁶, particularly since the embryo, at day 9 of gestation, has developed a heart and a circulatory system in which cells can migrate from yolk sac to embryo and to placenta and back.

The ability of placental cells from as early as day 9 of gestation to differentiate into mature B-cells *in vitro* offers exciting new experimental possibilities of probing the repertoire of variable regions of heavy and light chains, expressed very early in embryonic development, which may be close to or identical to

the repertoire carried in the germ line. If cellular contact between stem cells and 'environment' is needed for B-cell differentiation, then the rudiment of placenta may be one of the earliest sites on which B-cell differentiation occurs.

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Augmentation by interferon of human natural and antibody-dependent cell-mediated cytotoxicity

LYMPHOCYTES from normal individuals may have considerable levels of cytotoxic reactivity against tumour cells, mediated by NK (natural killer) cells¹. There are many similarities between NK cells and the effector cells (K cells) mediating antibody-dependent cell-mediated cytotoxicity (ADCC) against tumour target cells^{2,3}, and they may in fact be identical cells. *In vivo* inoculation of rodents with viruses^{4,5}, immunostimulants^{4,6-8}, tumour cells⁴ and other interferon inducers⁸ results in augmented NK activity. Interferon (IF) was shown to have a major role in rapidly boosting mouse NK activity, both *in vivo*^{9,10} and *in vitro*⁹. Trinchieri and Santoli¹¹ have suggested that IF could also augment human NK activity. However, the preparations used might have contained a variety of other lymphokines. The present study shows that IF can indeed augment human NK activity and also ADCC.

Peripheral blood mononuclear leukocytes (PBL) were collected from normal adult donors by centrifugation of heparinized blood on Ficoll-Hypaque gradients. Cytotoxicity was measured in a 4-h ⁵¹Cr-release assay, with K562 used as the target cell for NK, and Chang liver cells coated with rabbit IgG antibody as the target for ADCC². All experiments were carried out with several effector:target cell ratios, and activity was expressed in lytic units (LU) per 10⁷ cells, 1 LU being defined as the number of cells required to produce 30% specific cytotoxicity². Three different, partially purified, human IF preparations were used: lymphoblastoid IF (IF_{LB}), induced by Sendai virus (batch LNI/77/3A; 1.57 × 10⁶ U IF per mg protein); leukocyte IF (IF_{LE}), induced in human leukocytes by Sendai virus¹² (lot 1, 1.1 × 10⁶ U per mg protein); and fibroblast IF (IF_F), induced by poly I:C (3 × 10⁵ U IF per mg protein).

Two rabbit antibodies against human IF were used: anti IF_F (pool 29-33, anti-IF titre of 10³ per ml) and anti-IF_{LE} (ref. 13) (R24, titre of 10⁴ per ml). To evaluate the effects of the IF preparations, PBL (2-5 × 10⁶ per ml) were preincubated at 37 °C with IF (usually 10³-10⁴ U ml⁻¹) and then washed before

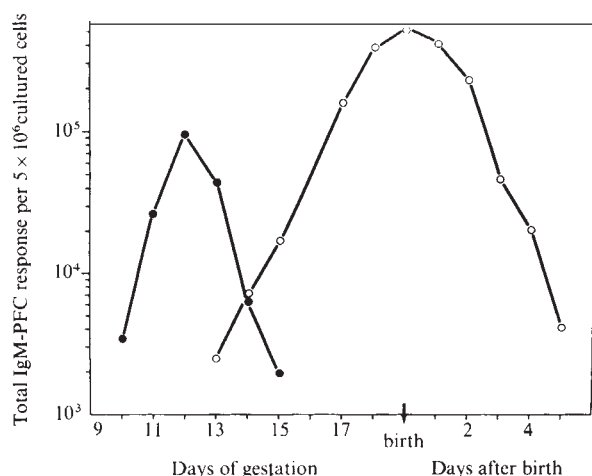


Fig. 2 LPS responsiveness of placenta (●) and fetal liver (○) (see ref. 9) cells taken in culture in the presence of LPS at different stages of development. IgM-secreting PFC responses were determined each day from the equivalent of day 15 of gestation until the equivalent of day 3 after birth of culture (see also Fig. 1). The sum of PFC determined at the different days is given as total PFC response.

Table 1 Effect of various human interferon preparations on cytotoxic activity of peripheral blood mononuclear cells

Donor	Treatment	NK activity			ADCC activity		
		% Cytotoxicity* 7:1	Lytic units per 10 ⁷ cells	% Of control lytic units	% Cytotoxicity† 7:1	Lytic units per 10 ⁷ cells	% Of control lytic units
1	Control	17.7	123	—	20.8	196	—
	IF _F (10 ⁴ U)	35.8	355	289	31.5	207	106
	IF _{LB} (10 ⁴ U)	37.2	405	329	29.2	179	91
	IF _{LE} (10 ⁴ U)	33.0	237	192	34.9	277	141
2		16.7:1			16.7:1		
	Control	13.3	16	—	16.9	16	—
	IF _F (10 ³ U)	26.6	64	400	20.4	39.4	250
	IF _F (10 U)	17.9	25.4	159	16.0	19	119
	IF _{LB} (10 ³ U)	21.3	33.3	208	35.8	104	656
	IF _{LB} (10 U)	20.9	32.5	203	25.7	76.6	480
	IF _{LE} (10 ³ U)	28.8	63	388	22.2	35.5	222
	IF _{LE} (10 U)	18.8	43	269	29.4	40	225

Interferon preparations: IF_F, interferon prepared from fibroblasts; IF_{LB}, interferon prepared from a lymphoblastoid cell line; IF_{LE}, interferon prepared from leukocytes. Preparations from donor 1 were treated for 1 h, those from donor 2 for 18 h.

* Baseline release of ⁵¹Cr from K562 target cells was 8.9%. Percentages given were obtained at one of the several effector: target cell ratios tested.

† Baseline release of ⁵¹Cr from antibody-coated Chang target cells was 10.4%. Percentages given were obtained at one of the several effector: target cell ratios tested.

Table 2 Summary of *in vitro* effects of interferon on NK- and K-cell activities of peripheral blood lymphocytes of normal donors

Treatment time (h)	Natural cytotoxicity			ADCC		
	Median	Range	No. expt >150% of control/total expt	Median	Range	No. expt >150% of control/total expt
1	160*	60–1626	11/22	141	91–455	3/12
18–20	168	75–292	7/11	164	90–494	8/11

* Activity after treatment as per cent of activity (calculated as lytic units per 10⁷ cells) in untreated control lymphocytes.

Table 3 Effect of anti-interferon on augmentation of cytotoxic activity by IF_{LB}

	Treatment	NK activity		ADCC activity	
		Lytic units per 10 ⁷ cells	% Of control	Lytic units per 10 ⁷ cells	% Of control
Expt 1	Control	13.3	—	44.4	—
	IF _{LB} (1,000 U)	40.9	297	70.5	159
	IF _{LB} + anti-IF _F (1:10)	2.1	5	0.4	<1
	IF _{LB} + anti-IF _F (1:40)	10.6	74	16.2	23
	IF _{LB} + anti-IF _{LE} (1:10)	0.0	<1	0.0	<1
	IF _{LB} + anti-IF _{LE} (1:40)	1.2	3	1.5	2
	Anti-IF _F (1:10)	0.1	<1	0.0	<1
	Anti-IF _{LE} (1:10)	0.2	1	0.0	<1
Expt 2	Control	70.2	—	36.7	—
	IF _{LB} (1,000 U)	170.0	242	51.2	148
	IF _{LB} + anti-IF _F (1:100)	86.1	122	31.6	86
	IF _{LB} + anti-IF _{LE} (1:100)	51.6	73	6.9	19
	Anti-IF _F (1:100)	69.3	99	14.9	41
	Anti-IF _{LE} (1:100)	39.5	56	1.1	3

Expt 1, preincubation of PBL at 37 °C for 18 h; expt 2, preincubation of PBL from another donor at 37 °C for 1 h.

testing for cytotoxicity. To evaluate the effects of anti-IF, various dilutions of antisera were added to the preincubation mixtures.

With some donors, augmentation in NK by IF was seen within a few minutes, with a peak at about 1 h (for example donor 2, Table 1). The three IF preparations gave parallel results, each showing effects down to 10 U ml⁻¹ or less. Table 2 summarises our overall results with several normal donors. NK activity of

some donors was boosted strongly, as much as 16-fold, whereas that of others was unaffected. The NK activity of a similar proportion of donors was appreciably (>150% of control LU per 10⁷ cells) boosted at 1 h and 18–20 h. In contrast, boosting of K-cell activity occurred more frequently after overnight incubation. The variations in responsiveness of different donors may be analogous to experiments in mice⁹, in which sensitivity to IF varied among strains and was influenced by previous *in vivo*

exposure to activating stimuli. Our data on NK activity agree fairly well with those of Trinchieri and Santoli¹¹, but it is not clear why they failed to detect the boosting of K-cell activity, as they preincubated their cells for 18 h.

To obtain further support for the role of IF as the active molecule in augmentation of human NK- and K-cell activities, we tested whether antibodies to IF could block the effects of IF. IF_{LB} was used for these experiments, so as to have an IF preparation from a source different from those used for immunisations, making it less likely that antibodies to other contaminating proteins would be responsible for the effects. Both antisera were able to block the boosting by IF_{LB} of NK- and K-cell activities completely (Table 3). In fact, these reagents caused a decrease to below unboosted levels. This was not due to toxicity, as after removal of the reagents, the cells could still be stimulated by IF and were normally responsive to the mitogenic effects of phytohaemagglutinin. Inhibition by IF-anti-IF complexes seemed unlikely as we have never been able to detect more than 5 U of IF per ml medium after 18 h incubation of PBL. However, to control further for this possibility, we compared the effects of anti-interferon with those of anti-bovine serum albumin (BSA), which would be expected to form complexes with the albumin in the fetal bovine serum (Table 4).

Table 4 Comparison of effects of anti-interferon and anti-bovine serum albumin on augmentation of cytotoxic activity by lymphoblastoid interferon

Treatment (18 h at 37 °C)	NK activity		ADCC activity	
	Lytic units per 10 ⁷ cells	% Of control	Lytic units per 10 ⁷ cells	% Of control
Control	111.1	—	175	—
IF _{LB} (1,000 U)	191.6*	172	230.9*	143
IF _{LB} + anti-IF _F (1:100)	95.5	86	25.7*	15
Anti-IF _F (1:100)	28.8*	26	5.0*	3
IF _{LB} + anti-BSA (1:100)	187.1*	169	123*	70
Anti-BSA (1:100)	99.7	89	38.4*	22

* Significantly different from untreated control, $P < 0.05$.

In contrast to the inhibition of NK activity and blocking by boosting by IF, the anti-BSA has no significant effects, although it did inhibit ADCC and its boosting by IF, presumably due to the known inhibitory effects of immune complexes on ADCC.

Because of the effect of complexes on ADCC and our inability to prepare F(ab')₂ fragments of anti-interferon (sufficient quantities of the reagents were not available), the effects of anti-interferon on boosting of ADCC by interferon cannot be readily interpreted. However, the marked effects of anti-interferon on NK activity seem to be specific and may indicate a normal role of IF in maintaining this effector cell function. As brief preincubation of IF with lymphocytes can induce activation of NK cells, it seems likely that surface-bound IF can lead to activation during the assay. The results with anti-IF suggest that some spontaneous NK activity results from activation during culture by IF bound *in vivo* or secreted during the assay.

The IF-activated effector cells had the same characteristics as NK cells. After incubation with IF for 18 h and before testing, monocytes were removed by passage of the cells over Sephadex G-10. No removal of cytotoxic activity against K562 or against antibody-coated Chang cells was observed after such treatment. Like NK and K cells², the IF-induced cytotoxic cells had Fc receptors for IgG, with complete removal of reactivity by adsorption on immune-complex monolayers.

The rapid and strong potentiation of cytotoxic effector cell activities by IF has important implications for *in vivo* resistance against tumour growth. These effects need to be seriously considered as possible mechanisms for the anti-tumour effects of a variety of immune adjuvants—as well as those of IF itself—which are being used in immunotherapy trials.

Human IF preparations were obtained from the NCI Interferon Working Group, and rabbit anti-IF antibodies from the Antiviral Substances Program, NIAID.

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Primary sensitisation and restimulation of human lymphocytes with soluble antigen *in vitro*

RESEARCH with human lymphocytes is restricted by ethical constraints on sensitising individuals *in vivo* before *in vitro* experiments. In humans specific restimulation of proliferative responses was first shown using allogeneic lymphocytes as antigen and has since been developed into the primed lymphocyte test¹. In a model similar to this test, again using cell-bound antigens, Newman *et al.*² showed sensitisation and restimulation by hapten-modified autologous cells. With soluble protein antigens and lymphocytes from donors who had been sensitised to these antigens *in vivo*, Levis and Dattner³ showed specific restimulation of proliferative responses, after the cells had been cultured in the presence of antigen for 10 or more days. We report here that, using a similar culture method, but cells from individuals who have not been sensitised *in vivo* to the relevant protein antigens, we have been able to obtain *in vitro* sensitisation and specific restimulation, sensitisation of the lymphocytes being determined by a subsequent specific proliferative response.

Lymphocytes were isolated by standard techniques from human peripheral blood and cultured for 10 d with the sensitising antigen. Cells were then washed and restimulated by culturing them for a further 2 or 3 d in the presence of the same or different antigens. Proliferative responses were measured by the incorporation of tritiated thymidine. Antigens used were key-hole limpet haemocyanin (KLH; Calbiochem), schistosomiasis soluble egg antigen (SEA), the synthetic polypeptides (T, G)-A--L (copolymer of L-(tyrosine, glutamic acid) coupled to D,L-alanine on a poly-L-lysine backbone, batch no. MC 9) and GAT (random copolymer of 60% glutamic acid, 30% alanine and 10% tyrosine, batch no. GAT 8) (both Miles Yeda), purified protein derivative (PPD) and tetanus toxoid (both RIV). Many individuals have been knowingly sensitised to these latter two antigens, but we emphasise that all data presented here have been obtained using combinations of antigens and cells which failed to respond in a direct proliferative assay.

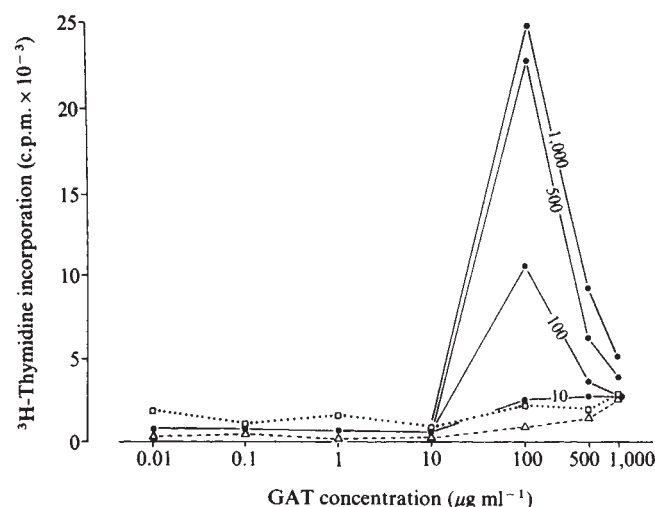


Fig. 1 Secondary responses of lymphocytes sensitised *in vitro* to GAT. Blood was obtained by venipuncture and lymphocytes were isolated by Ficoll-Hypaque density centrifugation (1.077 mg ml^{-1}). Cells from the interphase were washed 3 times in Hank's balanced salt solution and resuspended in RPMI 1640 supplemented with 30% AB serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin, and adjusted to 2×10^6 cells per ml. Of this cell suspension, 2.5 ml was mixed with 2.5 ml of the antigen diluted in RPMI 1640 (without serum) in a 25-cm bottle (Falcon no. 3013). Bottles were gassed with a 5% CO_2 /air mixture, closed and cultured for 10 d at 37°C in upright position. Approximately 1 in 10 of the cultures failed to retain an adequate pH and were discarded. Cells were then collected, adherent cells with a rubber policeman, washed once, and readjusted to 2×10^6 cells per ml in RPMI 1640 with 30% AB serum. Restimulation was carried out in duplicate or triplicate in flat-bottomed microtitre plates. A volume of 0.1 ml of the cell suspension was mixed with 0.1 ml RPMI 1640 (without serum) containing antigen. After 48 h tritiated thymidine was added, the cultures were collected 18 h later, and samples counted in a liquid scintillation counter. Seven different sensitising concentrations of GAT are shown on the abscissa. The second cultures of the sensitised lymphocytes were restimulated with: 10, 100, 500 or $1,000 \text{ μg ml}^{-1}$ of GAT ($\bullet$), 100 μg ml^{-1} of PPD ($\square$), or medium only ($\triangle$). When 10 μg ml^{-1} or less of GAT was used in the sensitising culture, all responses to GAT in the second culture were less than 1,000 c.p.m. and are not shown separately. The secondary responses of the GAT-sensitised lymphocytes to (T, G)-A--L in the concentrations 10, 100, 500 and $1,000 \text{ μg ml}^{-1}$ are also not shown. These responses were all in the range of the responses to PPD (with a maximum of 3,200 c.p.m.). In this experiment there was no evidence for cross-reactivity between GAT and (T, G)-A--L.

To obtain *in vitro* sensitisation, we found that the most critical parameter was the concentration of antigen during the first culture. Figure 1 shows an example of results obtained, in which GAT was used as antigen. With sub-optimal low concentrations of antigen no specific proliferative response was found in the second culture. In this experiment, 100 μg ml^{-1} GAT yielded optimal specific sensitisation, as shown by the highly significant response in the second culture to GAT compared with that to other antigens. Higher concentrations showed low, or even depressed, proliferative responses in the second culture, depending on the antigen used. In general, the background proliferative responses, that is, the responses to unrelated or no antigen in the second culture, increased with concentrations of antigen slightly higher than the optimal concentration in the first culture. The nature of this phenomenon is now being investigated. Finally, the concentration of antigen used in the second culture had to be varied but was less critical. The optimal concentration of antigen for the second culture has always been greater than the optimal sensitising concentration (Figs 1, 2). For sensitising cultures with PPD and tetanus toxoid, the optimal antigen concentrations were less than 1/10th of the optimal

concentrations routinely used by us for the direct lymphocyte transformation test with *in vivo* sensitised cells.

Figure 2 gives the results of an experiment in which cells were separately sensitised to three different antigens and restimulated with each of the three antigens. The data used were obtained with the optimal concentrations of each antigen. For all three antigens the proliferative response was significantly increased ($P < 0.01$, by analysis of variance on log transformed data) if the same antigen was used in the first as well as in the second culture. The lymphocytes in this and similar experiments were all negative in the direct lymphocyte transformation test with these antigens. Only the absence of current immunity can be concluded from a negative direct lymphocyte transformation test, and especially for tetanus toxoid a negative direct test does not exclude prior *in vivo* sensitisation. In further experiments, we used other antigens including synthetic polypeptides, for which it is much less likely that individuals have been sensitised *in vivo*. Table 1 shows the results of such experiments. In each experiment, at least two different antigens were used at several concentrations in the sensitising cultures. The lymphocytes from each culture were then restimulated with the relevant antigens. It was again shown that a specific proliferative response was only obtained using the same antigen in the second culture as had been used in the sensitising culture. Each experiment has been repeated at least three times with different donors and demonstrates our ability to sensitise unprimed human lymphocytes *in vitro* to different protein antigens.

We thus find that we can consistently sensitise lymphocytes from many different individuals to the antigens KLH, GAT, PPD and tetanus toxoid. Of 14 individuals tested with (T, G)-A--L and GAT, all responded to GAT whereas three failed to respond to (T, G)-A--L. These three also failed to show cross-sensitisation by GAT for response to (T, G)-A--L in the second culture, whereas 9 out of the other 11 showed such a reaction. For one of the non-responders the test has been repeated three times with different bleedings showing the same results. The genetic basis of this unresponsiveness to (T, G)-A--L is not known but is now under investigation. Restimulation after

Table 1 Proliferative response of human lymphocytes to restimulation *in vitro*

First culture	Antigen concentration (μg ml^{-1})		Response in c.p.m. per 10^5 cells $\pm$ s.d.
	Second culture		
KLH 1	KLH 100		*41,163 $\pm$ 823
	KLH 500		*40,311 $\pm$ 4,434
	SEA 100		6,232 $\pm$ 1,495
	SEA 500		6,835 $\pm$ 273
	—		6,413 $\pm$ 1,796
SEA 1	KLH 100		2,442 $\pm$ 170
	KLH 500		2,044 $\pm$ 141
	SEA 100		*5,234 $\pm$ 969
	SEA 500		*5,089 $\pm$ 1,007
	—		1,907 $\pm$ 303
(T, G)-A--L 500	(T, G)-A--L 500		*30,709 $\pm$ 5,527
	(T, G)-A--L 1,000		*31,651 $\pm$ 158
	GAT 500		18,177 $\pm$ 908
	GAT 1,000		19,614 $\pm$ 2,549
	—		7,302 $\pm$ 1,095
GAT 100	(T, G)-A--L 500		21,941 $\pm$ 1,316
	(T, G)-A--L 1,000		20,072 $\pm$ 1,003
	GAT 500		*33,528 $\pm$ 3,017
	GAT 1,000		*51,376 $\pm$ 2,055
	—		14,128 $\pm$ 2,684

Lymphocytes from one donor were cultured with different antigens in the first culture and restimulated as indicated. The results show the mean and standard deviation of duplicates. In this particular experiment, cross-reactivity between (T, G)-A--L and GAT which was found in nine out of eleven cases, is also shown. Each experiment was repeated at least three times with different donors and showed the same pattern of results.

* Results significantly different from controls ($P < 0.01$).

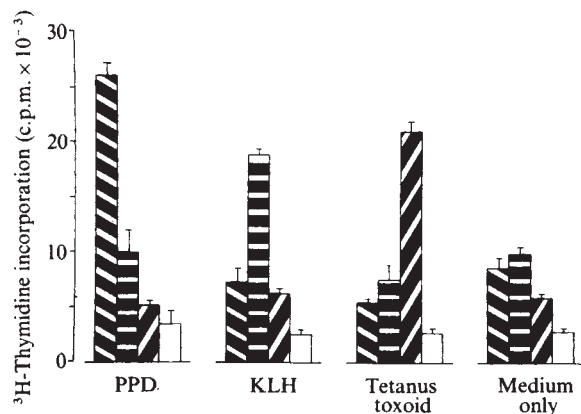


Fig. 2 Secondary responses of lymphocytes sensitised *in vitro* to PPD, KLH and tetanus toxoid. Lymphocytes were sensitised to four different concentrations of the antigens. Only data from the optimal sensitising concentrations are shown. These were $0.2 \mu\text{g ml}^{-1}$ of PPD (▨), $0.2 \mu\text{g ml}^{-1}$ of KLH (■), $10^{-4} \text{Lf ml}^{-1}$ of tetanus toxoid (▤) and medium alone (□). The optimal concentrations of antigen for restimulation were $50 \mu\text{g ml}^{-1}$ of PPD; $100 \mu\text{g ml}^{-1}$ of KLH or $10^{-1} \text{Lf ml}^{-1}$ of tetanus toxoid. In this experiment, the background proliferation in the second cultures was not influenced by the concentration of the irrelevant antigens. Second cultures were carried out in duplicate and results are shown $\pm$ s.d. Antigens used for restimulation are indicated on the abscissa. Antigens used for sensitisation are shown by the hatching of the bars.

sensitisation with SEA was not specific in 50% of the cases, possibly because this material is a complex of many antigens. It should be added that this culture method can also be used for sensitisation to insoluble antigens, and a study of the *in vitro* response to TNP-modified autologous cells is under way.

In vitro sensitisation to soluble antigens has been previously described by Thomas and Shevach⁴ using guinea pig cells. However, in their cultures, macrophages obtained from oil-induced peritoneal exudate cells were present. Comparable culture conditions for human cells would be difficult to obtain. An alternative assay for *in vitro* sensitisation, a primary *in vitro* antibody response, should be mentioned here. This method was described in humans by Dosch and Gelfund⁵, and elaborated to a further extent by UytdeHaag *et al.*⁶. Comparison of the two methods is difficult because lymphocyte proliferation has not been reported in their investigations, and we have not determined whether our cultures contain antibody-secreting cells.

Therefore, our data demonstrate that specific proliferative responses to soluble protein antigens can be induced *in vitro* using peripheral blood lymphocytes from donors who had not been immunised *in vivo* against these antigens. It is hoped that this assay will lead to a further understanding of the control of the immune response in man, and in particular the analysis of human immune response genes.

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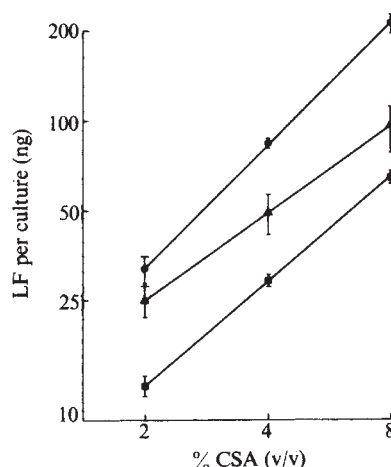
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Immunochemical quantification of *in vitro* neutrophilic granulocyte differentiation

In the presence of colony stimulating activity (CSA)¹⁻⁵, both normal and neoplastic cells of the neutrophilic granulocyte (neutrophil) and monocyte-macrophage cell series proliferate and differentiate in semi-solid and liquid cultures⁶⁻⁹. However, as conventionally performed, the clonal assay for CSA depends on cellular proliferation, and does not provide quantitative information on the role of CSA in differentiation. Lactoferrin (LF) is a specific protein marker for the neutrophil cell series¹⁰⁻¹², and among bone marrow cells is found exclusively in the specific granules of the maturing neutrophil^{13,14}. These granules first appear at the myelocyte stage of cytodifferentiation¹⁵. Thus, synthesis of LF is a biochemical event which may be used to quantify a specific state of neutrophil differentiation. We previously reported the purification of murine LF¹⁶, and its quantification by radioimmunoassay¹⁷, and also the development of a slide chamber method for culturing bone marrow cells in liquid medium¹⁸. We now report that in similar cultures, the net synthesis of LF is correlated in a dose-dependent manner with CSA concentration. To our knowledge, this is the first demonstration of a quantitative relationship at both the biochemical and cellular levels between CSA and a specific marker of neutrophilic granulocyte differentiation¹⁹.

Fig. 1 Dose-response relationship between the net synthesis of lactoferrin and the concentration of CSA in cultures of mouse bone marrow cells. Cells were obtained from 4-month-old male BALB/c mice from our breeding colony. A single cell suspension in Hank's balanced salt solution deficient in calcium and magnesium was layered over a solution of Ficoll-Hypaque (density 1.077 g cm^{-3}), and centrifuged at room temperature for 30 min at 400 g. Cells collecting at the interface were resuspended in RPMI-1640 culture medium containing 20% fetal bovine serum, and used to initiate cultures (1 ml, 1.25×10^4 cells) in four-chamber Lab-Tek microscope slide chambers¹⁸. Cultures were stimulated at time zero by the addition of various amounts of CSA from endotoxin-treated mouse lung²⁰, and were incubated at 37°C in a fully humidified atmosphere containing 7.5% CO_2 . Cultures were terminated at various times by carefully drawing off the medium and adding 0.5 ml of 0.01 M phosphate buffered saline, pH 7.4, containing 0.1% Triton X-100 and 0.3 M sodium chloride. The mixture was allowed to stand for 20 min at 4°C after which it was removed by aspiration. Aliquots of the aspirate were taken for quantification of LF by radioimmunoassay¹⁷. With the low initial cell inoculum used in these particular experiments (where correlation with microscopic observations was important) levels of LF on days 1 and 2 of culture were not sufficiently high to be detected by the radioimmunoassay. Each point is the mean of two to three replicates. The vertical bars indicate the range of values on day 3 (■), day 4 (▲), and day 5 (●).



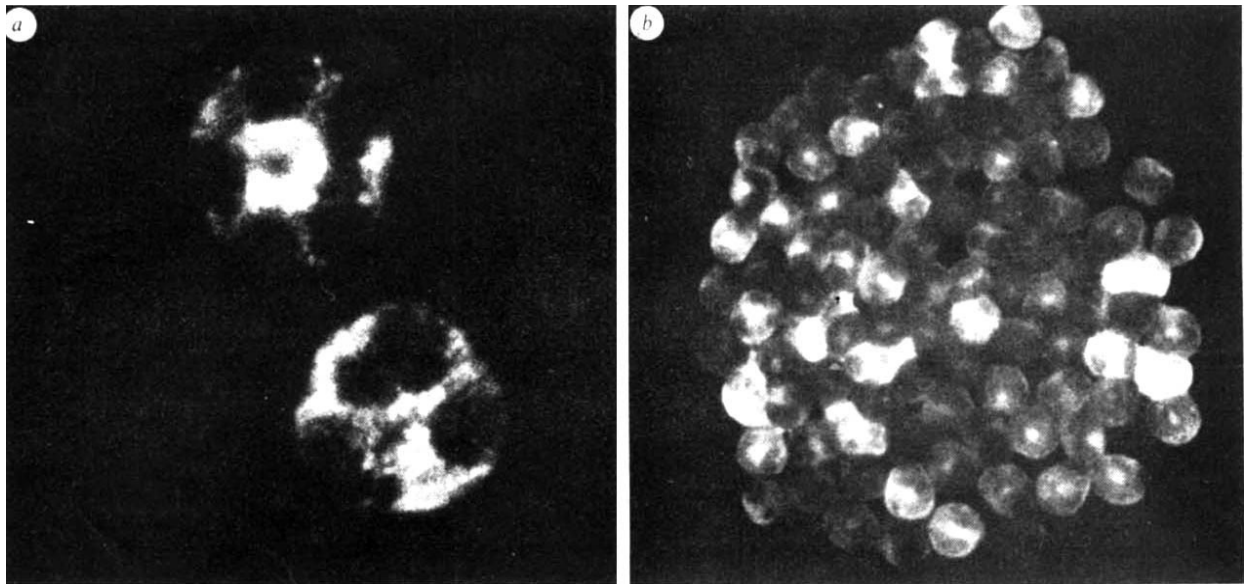


Fig. 2 Immunocytofluorescence of LF-containing, cultured murine bone marrow cells. After careful removal of the culture medium, wet slides were immediately centrifuged in a cytocentrifuge and cells were fixed in absolute ethanol containing 10% formalin¹⁸. Fixed cells were incubated with the fluorescein-labelled F(ab')₂ fragment of rabbit IgG raised against murine LF¹⁶. Slides were viewed using a Leitz Orthoplan fluorescence microscope equipped with a 100-W mercury lamp and epi-illumination. Photographs were taken with a Leitz Orthomat camera and Kodak Ektachrome ET135 film. *a*, Mature neutrophils showing a pattern of fluorescence characteristic of cells which have differentiated to the myelocyte stage or beyond ($\times 1600$); *b*, a single neutrophilic granulocyte clone displaying asynchronous differentiation as indicated by the presence of both LF-positive and LF-negative cells ($\times 400$).

Slide chamber cultures of murine bone marrow cells were stimulated by the addition of CSA from endotoxin-treated mouse lung²⁰. When examined between days 3 and 5, a linear dose-response relationship was observed between dose of CSA and the total amount of LF per culture (Fig. 1). In addition, it was evident from these data that the magnitude of the response between day 3 and day 5 increased approximately threefold as CSA concentration increased from 4 to 8%.

It was possible to extend these biochemical observations to the cellular level by examining cultured cells which had been fixed and exposed to a fluorescein-conjugated anti-LF antibody. LF-containing cells displayed a characteristic granular pattern of fluorescence (Fig. 2*a*). As shown in Table 1, there was a linear dose-response relationship between dose of CSA, the number of LF-containing cells, and the total number of cells per culture. The latter response (total cells) was more pronounced because the marrow cell population contained precursor cells which proliferated in response to CSA, but whose progeny did not contain LF (neutrophils less mature than myelocytes, and all cells of the monocyte-macrophage series).

The distinctive growth pattern of neutrophils offered an additional advantage for immunocytofluorescence studies. In the

liquid cultures, clones of neutrophils grew as discrete, tight, spheroidal cell groupings which could be easily identified through the first 3 days of culture. In these early cultures, we observed that within individual clones, the proportion of cells which contained LF varied from zero to unity, with some clones consisting of both LF positive and LF negative cells (Fig. 2*b*). This pattern of asynchronous differentiation within individual clones has also been observed in both normal and leukemic *in vitro* erythropoiesis^{21,22}. Note that with the cell concentrations used in the present experiments, cell crowding made interpretation of clonally-related events difficult beyond day 3 of culture. However, use of a lower initial cell inoculum (5 to 10×10^2 cells) resulted in sufficient spatial separation of individual clones to permit observation of clonally-related events beyond day 3. In these conditions, we observed that as tight clones matured, all cells eventually contained LF²³. We are currently investigating the effects of CSA concentration on the time course of LF synthesis in these clones composed exclusively of cells committed to neutrophilic granulopoiesis.

There is now good evidence that CSA preparations are heterogeneous with respect to biological activity, and that different species of CSA may govern the differentiation of neutrophilic granulocytes as distinct from monocytes and macrophages²⁴⁻²⁷. Furthermore, there is the possibility that some molecules of CSA might have either proliferative- or differentiative-associated functions while others might possess both of these activities. The assays for LF presented above should aid in the identification and purification of such postulated CSA species. Finally, the question as to whether CSA plays a direct part in neutrophil differentiation must remain open. The demonstration of a dose-response between CSA and net synthesis of a differentiation-associated protein such as LF is necessary but not sufficient proof of direct involvement, as the response might be secondary to an earlier, primary effect on neutrophil proliferation. However, studies on CSA-dependent synthesis of LF in the presence of appropriate inhibitors of DNA and RNA synthesis should provide further insight into this question.

In conclusion, we anticipate that the radioimmunoassay and immunocytofluorescent procedures for the detection and quantification of LF described here, both individually and in

Table 1 Effect of increasing concentration of CSA on the total number of LF-containing cells and on the total number of cells per culture

CSA concentration (μ l per ml culture)	No. of cells*	
	LF-containing	Total
4	1,326 $\pm$ 99	3,384 $\pm$ 261
16	2,347 $\pm$ 153	5,756 $\pm$ 206
64	3,188 $\pm$ 301	8,856 $\pm$ 643

* Details of the culture and immunofluorescence procedures are as described in the legends to Figs 1 and 2 except the initial cell inoculum was 1×10^4 cells (8% of the cells contained LF). In addition to cells committed to neutrophilic granulopoiesis, the total cell population contained cells of the monocyte-macrophage series which also proliferated in response to CSA. Cultures were terminated after 3 days, and all cells in each culture chamber were counted and classified by sequential observation with phase contrast and fluorescence microscopy ($\times 400$). Each point is the mean $\pm$ s.e.m. of four chambers.

combination, will prove useful in further defining the mechanism of action of CSA in the regulation of *in vitro* granulopoiesis.

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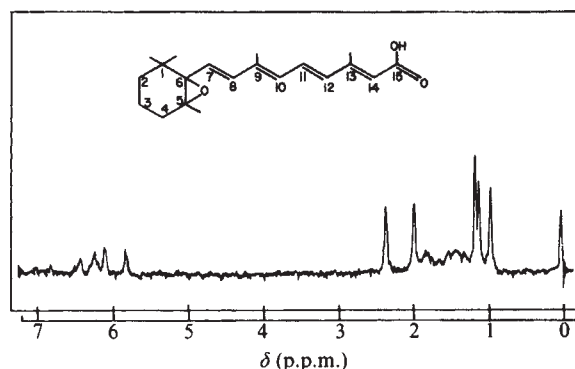


Fig. 1 The 60 MHz proton magnetic resonance spectrum of 5,6-epoxyretinoic acid. DCCl₃ containing 1% tetramethylsilane as internal standard was the solvent used in recording the above spectrum: δ (p.p.m.) 5.77–7.08 (6H, m, olefinic protons on side chain); 2.33 (3H, s, H₃C- on C-9); 1.97 (3H, s, H₃C- on C-13); 1.2–1.9 (6H, m, -CH₂- in ring); 1.13, 1.08 (6H, 2s, H₃C- on C-1); 0.92 (3H, s, H₃C- on C-5). This spectrum agrees with data presented by Vetter *et al.*¹¹ and is very similar to the spectrum reported for 5,6-epoxyretinal (ref. 12). The mass spectrum of 5,6-epoxyretinoic acid (melting point 153–154°C) contained an intense molecular ion (M , $m/e = 316$) and $M+1$ and $M+2$ peaks with intensities of 21% M and 2% M , respectively. No ions of higher m/e were observed. The UV absorption maximum found for this material (337 nm, ethanol) agrees with that reported for enzymatically produced 5,6-epoxyretinoic acid (337 nm, light petroleum)¹³.

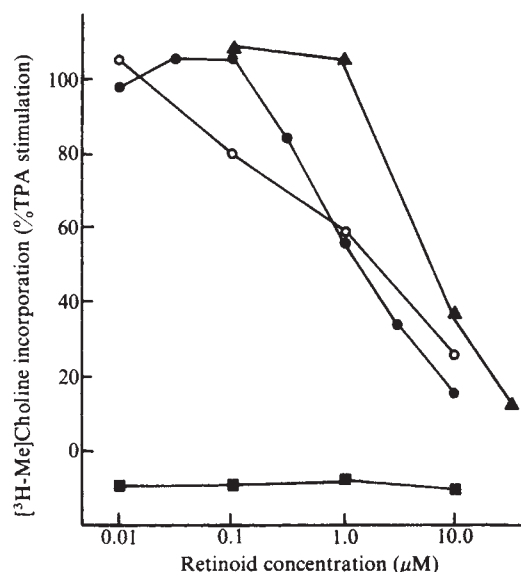


Fig. 2 Dose-response curve for the inhibition of [³H-Me]choline incorporation into the phospholipids of bovine lymphocytes. Lymphocytes were prepared from bovine retropharyngeal lymph nodes as described previously⁴, and were cultured in Eagle's HeLa medium supplemented with 10% bovine serum (BEHM) at a concentration of 5–10 × 10⁶ cells ml⁻¹ for 1 d before use. Cells were collected by centrifugation at 150g for 10 min and resuspended in Dulbecco's phosphate-buffered saline containing 5.6 mM glucose at 3 × 10⁶ cells ml⁻¹. Five-ml portions of cell suspension were then planted in 12-ml conical centrifuge tubes containing retinoic acid or the epoxide; after 15 min at 37°C, 2 μ Ci of [³H-Me]choline (69.5 Ci mmol⁻¹) and 50 pmol of TPA were added to each culture. Dimethyl sulphoxide, used as the solvent for TPA and the retinoids, was present at a final concentration of 0.55% (v/v) in all cases. After a 1-h incorporation period, the cells were collected and the phospholipids extracted and purified as described elsewhere². Radioactivity in the dried phospholipid extracts was quantitated by liquid scintillation spectrometry. Points represent the means of duplicates. ○, TPA plus retinoic acid; ●, TPA plus 5,6-epoxyretinoic acid; ▲, TPA plus 5,6-epoxyretinoic acid with medium containing 10% bovine serum; ■, 5,6-epoxyretinoic acid without TPA.

5,6-Epoxyretinoic acid opposes the effects of 12-*O*-tetradecanoylphorbol-13-acetate in bovine lymphocytes

12-*O*-TETRADECANOYLPHORBOL-13-ACETATE (TPA), the most potent of a series of tumour-promoting agents isolated from croton oil¹, has been found to be highly active in bovine lymphocytes as a stimulator of choline phospholipid metabolism², an inducer of ornithine decarboxylase³, and a co-mitogen when used in conjunction with phytohaemagglutinin (PHA)^{4,5}. These effects of TPA in the bovine lymphocyte system are antagonised by retinoic acid^{2,5}, an agent which opposes the tumour-promoting action of phorbol esters in mouse skin^{6,7} and inhibits chemical carcinogenesis in several other tissues⁸. However, the dose-response curves for retinoic acid in inhibiting these effects in lymphocytes are broad and extend over several orders of magnitude, suggesting that retinoic acid may have to be metabolised to be biologically effective in this system.

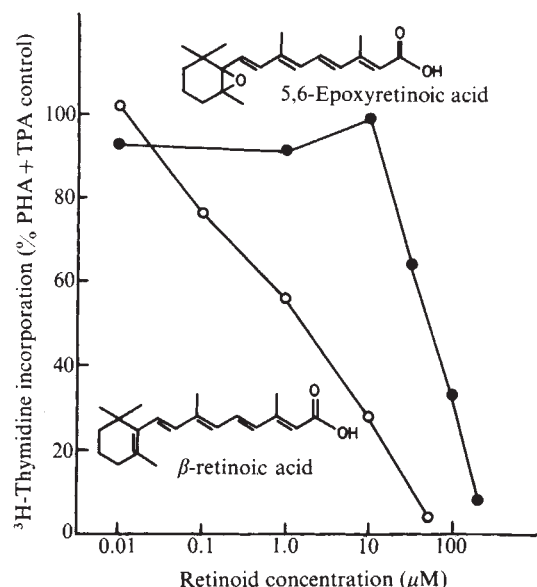


Fig. 3 Dose-response curves for the inhibition of lymphocyte mitogenesis by retinoic acid (○) and 5,6-epoxyretinoic acid (●). Cells were cultured for 1 d as described in the legend to Fig. 2. The lymphocyte cell density was then adjusted to 3×10^6 cells ml^{-1} in BEHM and 5-ml aliquots of the suspension were planted in conical 12-ml centrifuge tubes. PHA-P, used at a final dilution of 1:10,000, TPA (10 nM) and the retinoids were added to cultures concurrently. The cultures were gassed with 5% CO_2 in air and incubated at 37°C on a slant for 48 h. The cells were then pulse-labelled for 2 h with $2 \mu\text{Ci}$ per culture of ^3H -thymidine ($64.7 \text{ Ci mmol}^{-1}$). The incorporated radioactivity was assayed using a standard Whatman GF/C filter disk procedure¹⁵. Points represent the means of triplicate cultures.

The possibility that epoxidation might be involved in the activation of retinoic acid and related retinoids was suggested by two observations. First, 10,11-epoxymethylfarnesoate (insect juvenile hormone III), which bears the epoxide moiety at a position corresponding to the double bond in the ionone ring of retinoic acid, was found to oppose the actions of TPA in bovine lymphocytes and exhibited a much steeper dose-response curve⁹. Second, we have demonstrated that epoxidation at the 5,6-position of β -ionone strikingly increases its activity as an antagonist of the co-mitogenic action of TPA as well as the TPA stimulation of phospholipid metabolism¹⁰. We describe here the chemical synthesis and biological testing of the 5,6-epoxy derivative of retinoic acid as an antagonist of TPA action in bovine lymphocytes. We report that the introduction of an epoxide group at this position results in an enhanced ability to oppose certain effects of TPA in these cells.

Retinoic acid (2 mmol) was epoxidated by treatment with a 10-fold molar excess of monoperphthalic acid in ethyl ether (30 ml) at room temperature, and the reaction was monitored by thin layer chromatography on silica gel, with 20% ethanol in light petroleum (boiling point $40-60^\circ\text{C}$) as the eluant. Within 0.5 h, all the retinoic acid had been consumed, with the formation of a single more polar product, as shown by thin layer chromatography. This reaction mixture was extracted twice with two volumes of aqueous NaHCO_3 (2% w/v) and washed with two volumes of water. When the organic phase was taken to dryness under vacuum, the product crystallised spontaneously (1.7 mmol); the latter was recrystallised from ethyl ether/light petroleum and characterised as 5,6-epoxyretinoic acid. The structure verification of racemic 5,6-epoxyretinoic acid is presented in Fig. 1.

As noted above, TPA accelerates the incorporation of choline into the phospholipids of bovine lymphocytes, and this response is antagonised by retinoic acid². Figure 2 shows that 5,6-epoxyretinoic acid is also an active antagonist of this process and that

when an effective concentration has been reached it exhibits a steeper dose-response curve than retinoic acid. Although other explanations of the data are possible, this observation is consistent with the hypothesis that epoxidation of the 5,6-double bond may be involved in the metabolic activation of retinoic acid. This possibility is strengthened by the report by Napoli *et al.*¹⁴ that 5,6-epoxyretinoic acid is a major retinoic acid metabolite in the intestinal mucosa of the rat.

Several additional points should be made concerning the data presented in Fig. 2. First, it seems unlikely that the inhibitory action of the epoxide is merely a manifestation of toxicity, as it does not affect the basal level of [^3H -Me]choline incorporation, and second, when 10% serum was included in the incubation medium the effective inhibitory range of epoxide concentrations was shifted upward by an order of magnitude. This observation suggests that serum and certain cellular components may sequester or facilitate the inactivation of low levels of 5,6-epoxyretinoic acid and thus account for the shoulder on the dose-response curve. This same situation was encountered in earlier experiments in which a similar serum effect on the effective inhibitory concentrations of 5,6-epoxy- β -ionone was observed¹⁰.

In Fig. 3, the contrast of broad- compared with steep-sloped dose-effectiveness curves for retinoic acid and the epoxide is clearly shown in the inhibition of the mitogenic response. As reported previously, retinoic acid inhibits TPA-mediated co-mitogenesis over a broad range of concentrations⁵; however, it

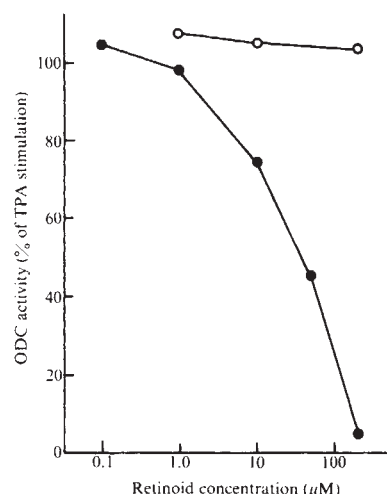


Fig. 4 Dose-response curves for the inhibition of ornithine decarboxylase (ODC) induction by retinoic acid (○) and 5,6-epoxyretinoic acid (●). Lymphocyte cultures were planted as described in the legend to Fig. 3. Eighteen hours after the concurrent addition of PHA, TPA, and retinoids to the cultures, the cells were collected and washed twice with cold 0.9% NaCl. The lymphocytes were then suspended in 0.3 ml of 50 mM sodium phosphate buffer (pH 7.2) containing 0.1 mM EDTA, and disrupted by four cycles of freezing and thawing. After centrifugation of the disrupted cells at $2,000g$ for 15 min, a clear supernatant was obtained. Ornithine decarboxylase activity in this supernatant was assayed by measuring the release of $^{14}\text{CO}_2$ from DL-[1- ^{14}C]ornithine hydrochloride ($49.9 \text{ mCi mmol}^{-1}$). The assay mixture contained 35 mM sodium phosphate (pH 7.2), 0.2 mM pyridoxal phosphate, 4 mM dithiothreitol, 1 mM EDTA, 0.4 mM L-ornithine containing $0.5 \mu\text{Ci}$ of DL-[1- ^{14}C]ornithine hydrochloride, and 100 μl of lymphocyte supernatant in a total volume of 0.25 ml. Incubation of the assay mixture at 37°C was carried out in 15-ml corex tubes equipped with centre well assemblies and capped with serum stoppers. After 1 h, the reaction was stopped by the addition of 0.5 ml of 2 M citric acid; however, incubation was continued for one more hour to ensure complete absorption of $^{14}\text{CO}_2$ by the ethanolamine and methoxyethanol (0.2 ml; 2:1) in the centre well. Finally, the contents of the centre well were transferred to a vial containing 10 ml of toluene-based scintillation fluid and 2 ml ethanol. Radioactivity was measured by liquid scintillation spectrometry. Points represent the means of triplicate cultures.

can be seen that retinoic acid epoxide displays a steep dose-response curve, as anticipated for the active inhibitory species. The fact that higher concentrations of the epoxide, as compared with the parent compound, are required to achieve inhibition is thought to reflect the above noted difficulty in delivering the chemically reactive epoxide to the appropriate cellular sites rather than an inherently lower activity of this derivative. Again, toxicity does not seem to be the basis of this inhibitory action of 5,6-epoxyretinoic acid, for 48 h of exposure to this agent does not affect the lymphocyte viability, as measured by the trypan blue exclusion test.

In a third series of studies, we have tested the ability of 5,6-epoxyretinoic acid to inhibit the induction of ornithine decarboxylase by TPA. Ornithine decarboxylase, the rate-limiting enzyme in polyamine biosynthesis, is among the earliest and most thoroughly studied responses induced by the application of TPA to mouse skin¹⁶. The TPA induction of this enzyme, which is prevented by retinoic acid, has provided a useful test system for assessing several retinoid analogues as biological antagonists of TPA in mouse skin. In PHA-treated lymphocytes, retinoic acid also blocks the TPA-induced ornithine decarboxylase activity; however, it must be added to cell cultures before TPA to exert this effect³. This temporal dependency is reflected in Fig. 4, in which the induction of ornithine decarboxylase by TPA proceeds unhindered by concurrently added retinoic acid. However, in these same conditions, 5,6-epoxyretinoic acid is a highly effective inhibitor of ornithine decarboxylase induction (Fig. 4).

The present results do not prove the concept of metabolic activation of retinoic acid, but they are consistent with the suggestion that an activation process may involve epoxidation at the 5,6-position. At the very minimum, it can be stated that epoxidation of retinoic acid at the 5,6-position is a tolerated modification of retinoic acid for activity of this agent as an antagonist of certain effects of TPA in the bovine lymphocyte system.

Thus, although bovine lymphocytes have not been used in tumour-promoting studies with phorbol esters or in anti-tumourigenic studies with retinoids, the present results and our earlier findings^{2-5,9,10} clearly demonstrate that they are a good indicator system for studying the modulation of metabolic processes by these two classes of agents. The responsiveness, the ready availability of the cells, and the ease of staging prompts their further use in studies of the molecular mechanisms by which these agents act.

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Migrating epidermis produces AB₂ collagen and requires continual collagen synthesis for movement

EPITHELIAL CELLS demonstrate remarkable motility during the *in vivo* processes of wound healing, organogenesis and neoplastic invasion¹. We have been interested in the control of epithelial motility. From earlier studies we know that *in vitro* epithelial movement requires the continual presence of a serum component^{2,3} and a proper substratum⁴. In culturing dissociated epithelial cells, collagen-coated dishes have been found to provide a better substratum than non-coated surfaces⁵. As studies in other laboratories had suggested that epithelium produces collagen⁶⁻⁸, it seemed appropriate to investigate the role of collagen in epithelial movement. Reported here are morphological and metabolic studies of a well defined tissue culture system of skin explants involving both epithelial outgrowth and epiboly. The observations support the possibility that the continual synthesis of collagen is necessary for epithelial cell movement.

The *in vitro* system we have used in measuring epithelial movement has been described elsewhere in detail^{2,3}. Briefly, measured mouse skin explants are laid on plastic Petri dishes (outgrowth) and covered with medium (Dulbecco's modified Eagle's medium, DME; Gibco) plus human serum (5 mg ml⁻¹) or suspended in the medium (epiboly). At intervals, the maximal radial outgrowth or the extent of epiboly are measured. In earlier experiments we established that epithelial spread is virtually independent of DNA synthesis and thus cell division; the outgrowth measured is therefore apparently due predominantly to epithelial cell motility. Although the explant contains dermal elements the outgrowth is essentially, if not completely, epithelial, as demonstrated earlier^{2,3}.

Our initial questions were: does migrating epithelium of the outgrowth and epiboly systems contain collagen? If so, what types of collagen are present in the migrating epidermis? Using affinity-purified antibodies to collagen (I, III, IV and AB₂) and an indirect immunofluorescence (IF) assay, we found that both stationary and migrating epidermis contain type AB₂ collagen (Fig. 1i, 1j). Types I (Fig. 1c, 1d) and III (Fig. 1e, 1f) are found exclusively in the dermal portion of the outgrowth. Type IV (Fig. 1g, 1h) is localised to the junction of the dermis and the stationary epidermis, but not beneath the migrating epidermis.

If both migrating and stationary epidermis synthesise AB₂ collagen, as the IF studies suggest, proline incorporation into these cells should be demonstrable, as proline and hydroxyproline constitute 20% of the amino acid residues of AB₂ collagen⁹. The autoradiography of ³H-proline-pulsed explants (Fig. 1a, 1b) shows silver grains over the cytoplasm of the migrating and stationary epidermis of both the outgrowth and the epiboly assays. Proline incorporation was present diffusely over the epithelial cell cytoplasm and grain density was not greater at the growing tip or the basement membrane regions. As essentially no proline incorporation was present in the dermis at any time throughout the experiment, we assume that proline is incorporated by the epithelium and not transferred to the epithelium from the dermis.

The synthesis of collagen by the epithelium was tested first by hydroxyproline synthesis and incorporation (a product of

Table 1 Epithelial spreading in the presence of the proline analogue L-azetidine-2-carboxylic acid

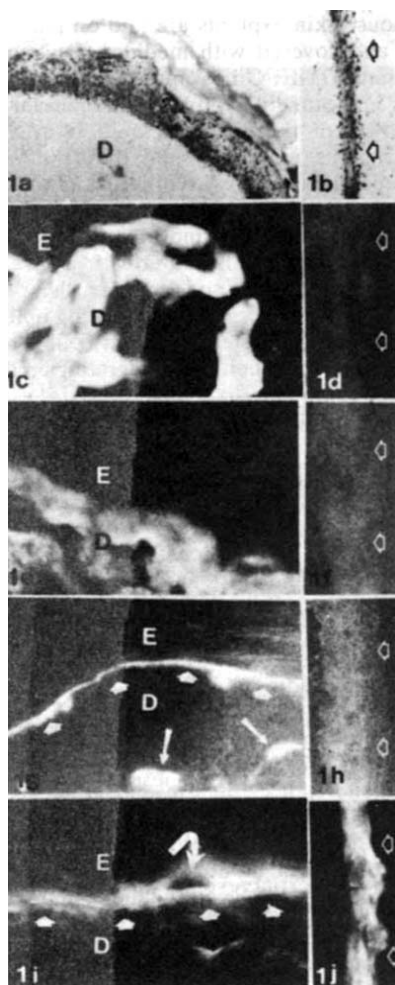
Experimental conditions	Linear outgrowth at 3 d (μm)	% Of control	Epiboly (%)
DME + HS (control)	465 $\pm$ 34 (35)	100	96 $\pm$ 4 (9)
DME + HS + cycloheximide (1 $\mu\text{g ml}^{-1}$)	0 $\pm$ 0 (5)	0	Not done
DME + HS + cycloheximide (5 $\mu\text{g ml}^{-1}$)	0 $\pm$ 0 (12)	0	Not done
DME + HS + LACA (1 $\mu\text{g ml}^{-1}$)	527 $\pm$ 65 (6)	113	Not done
DME + HS + LACA (10 $\mu\text{g ml}^{-1}$)	48 $\pm$ 15 (32)	10	5 $\pm$ 1 (11)
DME + HS + LACA (100 $\mu\text{g ml}^{-1}$)	0 $\pm$ 0 (11)	0	0 $\pm$ 0 (9)
DME + HS + LACA (10 $\mu\text{g ml}^{-1}$) for 1 d and then DME + HS for 2 d	273 $\pm$ 33 (10)	113*	Not done
DME + HS + proline (200 $\mu\text{g ml}^{-1}$)	459 $\pm$ 31 (16)	99	100 $\pm$ 0 (12)
DME + HS + LACA (10 $\mu\text{g ml}^{-1}$) + proline (200 $\mu\text{g ml}^{-1}$)	442 $\pm$ 52 (12)	95	95 $\pm$ 4 (11)
DME + HS + LACA (100 $\mu\text{g ml}^{-1}$) + proline (200 $\mu\text{g ml}^{-1}$)	254 $\pm$ 27 (13)	58	89 $\pm$ 6 (8)

Outgrowth and epiboly experiments were set up as previously described^{2,3}. Linear outgrowth is recorded as the maximal radial spread of epithelium measured from the explant edge. The data are recorded as the mean $\pm$ s.e.m. (no. of observations). Outgrowth in the presence of DME + HS for 3 d was used as the control in calculating the percent of outgrowth except for the recovery experiment (indicated by asterisk), where outgrowth after 2 d in DME + HS was used as the control. Epiboly is expressed as the mean per cent of the lower explant covered by epithelium $\pm$ s.e.m. (no. of observations) after 2 d of incubation. HS was prepared as previously described^{2,3}.

collagen biosynthesis), and second by incorporation of ^3H -proline into antigen precipitable by monospecific collagen antibodies. The synthesis of hydroxyproline by the epidermis was tested using epiboly explants incubated for 24 h in the conditions described in the legend to Fig. 1; however, in these experiments the ^3H -proline pulse was given for 24 h. The epidermis was removed from the dermis after incubating in hot water (55°C) by the method of Marrs and Voorhees¹⁰. The epidermis was then hydrolysed and the hydrolysate analysed by the thin-layer chromatographic technique of Myhill and

Jackson¹¹. Approximately 4% of the applied counts co-migrated with the hydroxyproline standard (H6002 Sigma).

To test for the incorporation of proline into collagen antigen, ^3H -proline-pulsed explants (grown as described in the legend to Fig. 1) were homogenised and extracted in 0.1 M acetic acid (24 h at 4°C). The supernatant was dialysed (0.01 M acetic acid) and lyophilised. The dried collagen was suspended (0.01 M acetic acid to 100 $\mu\text{g ml}^{-1}$) and dialysed into neutral buffer (0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl). To 0.2 ml of this solution, 0.1-ml aliquots of AB₂ collagen antibody (or normal

**Fig. 1** Autoradiography and collagen immunofluorescence of epithelial

outgrowth and epiboly. Using our previously described *in vitro* explant system^{2,3}, 0.2-cm disks of 6–10-week-old mouse ear skin were grown for 3 d on 0.22 μm sterile Millipore filters in DME containing 5 mg ml^{-1} dialysed and lyophilised human serum in 95% air, 5% CO_2 and saturated water at 37°C. By 3 d appreciable epithelial outgrowth had extended from the explant over the filter. For the epiboly experiments, using the same tissue culture set-up, skin disks were floated on identical medium for 48 h. For both sets of experiments, at the indicated times the tissues were removed, fixed in 10% buffered formalin and prepared for light histological examination as previously described^{2,3}. Autoradiography was carried out as described elsewhere^{3,3}; the explants were pulsed for 2 h in medium containing L-[2,3- ^3H]proline (3 $\mu\text{Ci ml}^{-1}$) (NEN, specific activity 20 Ci mmol^{-1}). Types I and III collagen were isolated from rat skin and types I, III and AB₂ collagen prepared from human placental membranes⁹ by previously described methods. Type IV collagen was prepared from EHS sarcoma³⁶. The collagens were judged pure by SDS-polyacrylamide gel electrophoresis of α chains (5%), CNBr peptides (7.5%) and amino acid composition. Rabbit antibodies were purified by cross-absorption on Sepharose affinity columns containing all other collagen types, followed by absorption affinity chromatography on a column containing the collagen type against which the serum was raised. They were of high titre (1:25,000 to 1:100,000) and specific for the collagen against which they were raised, as tested by haemagglutination, haemagglutination inhibition and/or radioimmunoassay^{24,37–40}. Immunofluorescence (IF) staining and microscopy of frozen sections of outgrowth and epiboly tissues were done using standard techniques with fluorescein-labelled sheep anti-rabbit IgG. Immunological cross-reactivity between mouse and rat collagens (I and III) and between human and mouse collagen (AB₂) was demonstrated by tissue immunofluorescence and haemagglutination inhibition (type I and AB₂) and radioimmune assay (type III). 1a, Autoradiography of outgrowth, epithelium over dermal plug (magnification $\times 500$); b, autoradiography of epithelial outgrowth (magnification $\times 500$). IF using antitype I collagen antibody of skin disk (1c) (the centrally lying non-fluorescent structure is a hair follicle) (magnification $\times 500$), and epithelial outgrowth (1d) (magnification $\times 500$). IF using antitype III collagen antibody of skin disk (1e) (magnification $\times 500$) and epithelial outgrowth (1f) (magnification $\times 500$). IF using anti-type IV collagen antibody of skin disk (1g) (magnification $\times 250$) and epithelial outgrowth (1h) (magnification $\times 500$). IF using anti-type AB₂ collagen antibody of skin disk (1i) (here the label E is above the epidermis which fluoresces at all levels) (magnification $\times 500$), and epithelial outgrowth (1j) (magnification $\times 500$). E, Epidermis; D, dermis; arrowheads, epithelial basement membrane; arrows, skin appendages; curved arrow, epithelial cell nucleus; open arrowheads, epithelial outgrowth. All fluorescence could be specifically inhibited by preincubation of antibody with 2.5 μg of the collagen being assayed but not by addition of other collagens.

rabbit serum) were added and the solution then incubated for 24 h (4 °C). At this time 0.2 ml of sheep anti-rabbit IgG anti-serum was added followed by an additional 24 h incubation (4 °C). The AB₂ collagen antibody precipitated 15% of the acetic acid-extractable counts.

If migrating epithelium produces collagen, as the above studies suggest, is its continual synthesis necessary for epithelial movement? Inhibition studies testing this possibility are described in Table 1. No outgrowth occurred in the presence of the general protein synthesis inhibitor, cycloheximide, at concentrations of 1 and 5 µg per ml of medium. This observation agrees with the epiboly experiments of Gibbins¹². Using the proline analogue L-azetidine-2-carboxylic acid (LACA), which affects collagen production^{13,14}, outgrowth and epiboly were studied. At 1 µg ml⁻¹ LACA, no inhibition of outgrowth was found, whereas at 10 µg ml⁻¹ and 100 µg ml⁻¹, inhibition was 90% and 100%, respectively. Removal of the inhibitor after 24 h led to the complete recovery of outgrowth. Adding L-proline to the medium (200 µg ml⁻¹) completely reversed the inhibition at 10 µg ml⁻¹ of LACA and partially corrected the inhibition at 100 µg ml⁻¹ LACA (Table 1). These experiments support the idea that the LACA effect is competitively inhibited by L-proline and that a proline-rich protein (perhaps collagen) is necessary for movement.

It is easiest to interpret an inhibitor experiment if the inhibition can be specifically reversed. If the proline-rich protein collagen is necessary for movement, it should theoretically be possible to observe movement in the presence of LACA if collagen were added back, that is, if collagen-coated dishes were used in the presence of LACA (Fig. 2). As observed in earlier studies^{2,3} on a plastic substratum, no epithelial outgrowth is seen before 24 h, but thereafter outgrowth is linear from 1 to 3 d (Fig. 2a, continuous line). As seen in Fig. 2a, in the absence of collagen, outward spreading of epithelium abruptly stopped after the addition of LACA (100 µg ml⁻¹) to the medium. No outgrowth was seen if LACA was added at day 1 and no increase in outgrowth was seen if it was added at day 2. On collagen-coated dishes (Fig. 2b) the effect is only quantitatively different. In the absence of inhibitor and presence of collagen-coated substratum (types I, III, IV and AB₂), outgrowth at 3 d is 59% greater than that on non-coated dishes (Student's *t* test *P* < 0.005). If LACA was added at zero time or at 2 d, no further outgrowth occurred despite the collagen substratum. Adding the inhibitor to the outgrowth experiments after the epithelium had made contact with the substratum or to the epiboly experiments at any time caused the cessation of epithelial movement. Adding the collagen back in solution (types I, III, IV or AB₂) (50–100 µg ml⁻¹) did not restore motility to outgrowth or epiboly experiments in the presence of LACA.

In these studies we have demonstrated: (1) that migrating epithelium contains type AB₂ collagen but not types I, III and IV; (2) that migrating epithelium synthesises and incorporates hydroxyproline and actively incorporates proline into an antigen precipitable by antibody monospecific to type AB₂ collagen; (3) that the proline analogue LACA reversibly inhibits epithelial migration. One interpretation of these results is that migrating epithelium synthesises and contains type AB₂ collagen and that collagen production (synthesis and secretion) is necessary for movement. Although it is the simplest conclusion for these results, we do not have direct chemical evidence that collagen synthesis is necessary for movement. If only the presence of collagen in the extracellular environment were sufficient to support migration, the 'add-back' experiments (collagen-coated dishes and collagen-containing media) should have overcome the LACA effect. The failure to restore motility in these experiments in the presence of LACA may indicate a more complex relationship. First, it may be possible that other proline-containing proteins are necessary for movement. We think this is unlikely not only because the cells contain collagen, but also because the system is very sensitive to inhibition (10 µg of LACA is inhibitory) and therefore the necessary proteins must be proline-rich (collagen-like). Second, it is possible that

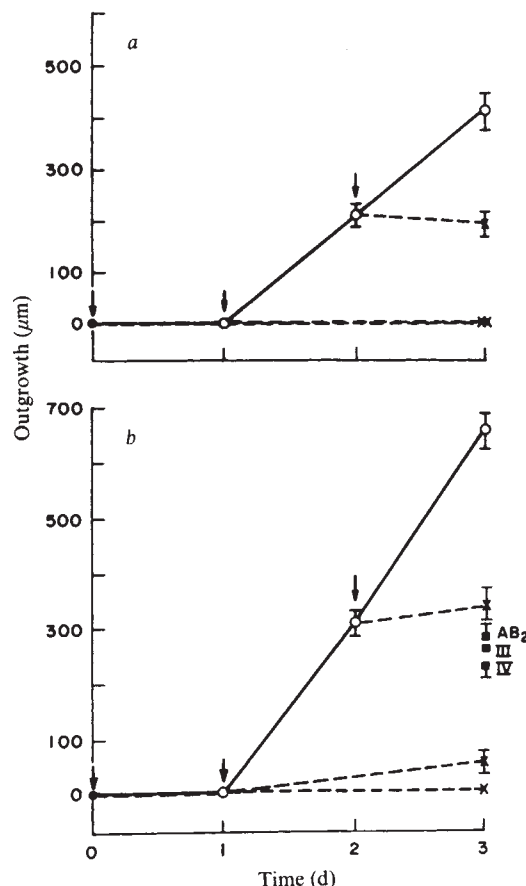


Fig. 2 Epithelial outgrowth on non-coated (a) and collagen-coated (b) dishes. As described for Fig. 1 and Table 1, skin explants were laid down on Petri dishes and radial outgrowth measured over a 3-d period. ○—○, DME+HS (5 mg ml⁻¹); x---x, DME+HS (5 mg ml⁻¹)+LACA (100 µg ml⁻¹). LACA addition is indicated by arrows. Each point represents the mean of 6–14 experiments ± s.e. In b, the squares represent the final outgrowth after LACA (100 µg ml⁻¹) was added at day 2 to experiments with type III, type IV and type AB₂ collagen-coated dishes. All other points are experiments with type I-coated dishes. The dishes were prepared by the method of Karasek and Charleton⁵ and the collagen concentrations were 50 µg cm⁻².

moving epithelial cells require the continual production of collagen which is cell-surface-associated or substratum-coated in a specific way which we have not been able to reproduce in the add-back experiments.

Collagen has been sought in epithelium before, although to our knowledge this is the first demonstration of type AB₂ collagen in epithelium. We were surprised that migrating epithelium did not contain type IV collagen, as epithelium is believed to produce its own basement membrane^{15–20}. This observation is compatible with other studies which report that basement membrane material is not morphologically present below migrating epithelium during the initial stages of movement^{15,21,22}. It is notable that AB₂ collagen has been found to be a component of some epithelial basement membranes^{23,24}.

LACA, a cyclic imino-acid plant extract, has been found to be competitively incorporated into proteins in place of proline²⁵. Its incorporation into collagen has been demonstrated²⁶ and the abnormal collagens formed seem to be inadequately secreted by the synthesising cell^{13,14}. The synthesis of an abnormal collagen and its inadequate secretion may explain our results in the presence of LACA. In contrast to the complete reversal by proline at low LACA concentrations (10 µg) (LACA/proline ratio of 1:20), only partial reversal was found at higher concentrations (100 µg) (LACA/proline ratio of 1:2) (Table 1). The latter case may be due to the production of abnormal collagen secondary to significant LACA incorporation²⁶ even in the presence of proline.

The specific role of AB₂ collagen in epithelial migration is still unknown. Other investigators have also found that collagen-coated substrata have a dramatic effect on various tissue culture systems (fibroblasts, mammary gland epithelium, hepatocytes, monocytes, corneal epithelium). The effect has included increased cellular adherence^{5,27}, differentiation^{28,29} and protein synthesis²⁹. Therefore, the observed enhancement of epithelial migration when explants are grown on collagen substrata (regardless of type) is not surprising. This phenomenon may represent a general charge or ionic effect rather than a specific property of collagen³⁰. Recently, Kleinman *et al.*³¹ and Murray *et al.*³² have demonstrated in short-term cultures that dissociated epithelial cells attach preferentially to type IV collagen-coated dishes. As our studies deal with epithelial cell sheets and relatively long-term migration patterns, a meaningful comparison between these studies cannot be made.

These experiments support the hypothesis that migrating epithelium contains AB₂ collagen and must liberate this collagen for continual movement. By understanding the interaction of epithelial cells and collagen metabolism in this system, we hope to gain insight into the control of epithelial movement in particular and, perhaps, cell movement in general.

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Survival and development of ciliary ganglion neurones grown alone in cell culture

NEURONAL cell death is a conspicuous part of development for many neuronal populations in the vertebrate nervous system^{1,2}. Although little is known about the mechanisms that control neurone death, it seems that interactions with the postsynaptic target tissue are important. Thus, for the chick ciliary ganglion (CG), Landmesser and Pilar showed that half of the neurones present die between days 8 and 13 in the embryo. Before cell death, all the neurones extend processes to the periphery. Ablating the periphery early in development causes more than 90% of the neurones to die at the normal time³. We have previously shown that nearly all the neurones from chick CG, including those destined to die *in vivo*, can survive and develop in cell culture when ganglia from 8-d-old embryos are dissociated and grown with skeletal myotubes⁴. Many of the neurones innervate myotubes in these conditions. Are neuromuscular synapses necessary to prevent the death of ciliary ganglion neurones? We report here that they are not. Culture medium conditioned by contact with heart or skeletal muscle cells permits complete long-term survival of the dissociated neurones grown in the absence of other cell types. The neurones develop levels of choline acetyltransferase (CAT) activity comparable to those obtained for neurones grown with myotubes.

Ciliary ganglia from 7–8-d-old chick embryos (Hamburger–Hamilton stages 32–34) were dissociated with trypsin as previously described⁴. The cells were plated in collagen-coated,

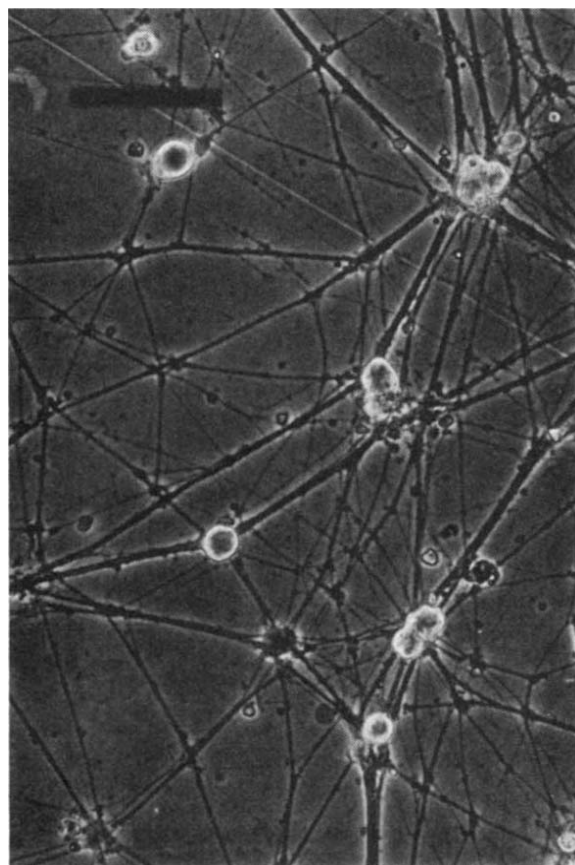


Fig. 1 Dissociated CG neurones after 3 weeks in culture. CG neurones dissociated from 8-d-old embryonic ganglia were maintained in heart-conditioned medium for 3 weeks. The photo was taken with phase contrast optics. Calibration bar: 100 μ m.

Table 1 Culture medium requirements for survival of CG neurones

Culture medium	Neuronal survival (neurones per ganglion equivalent)	CAT activity (fmol ACh per neurone per h)
Conditioned + emb. ext.	6,900 ± 400	220 ± 24
Conditioned + emb. ext. + cytarabine	5,400 ± 200	ND
Unconditioned + emb. ext.	6,600 ± 100	370 ± 30
Conditioned	5,500 ± 400	170 ± 20
Unconditioned	0	ND

Cultures were seeded with ~6,500 neurones (1 ganglion equivalent) from 8-d embryos and were maintained for 11 d in the indicated medium. The cultures were then examined for surviving neurones and for CAT activity. Each value represents the mean ± s.e.m. of at least 7 cultures from 2 or more cell platings. Heart cell cultures were used to condition the medium where applicable. For these experiments only, embryo extract (emb. ext.) was not present unless indicated. Cultures treated with cytosine arabinoside (cytarabine) were exposed to a concentration of 10^{-5} M only during the first 2 d of the 11-d period. ND, not determined.

16-mm Falcon tissue culture wells at a density of 1 ganglion equivalent (~6,500 neurones) per well. Cultures plated at lower cell densities yielded variable results. Standard culture medium consisted of Eagle's minimal essential medium supplemented with 10% heat-inactivated horse serum (Microbiological Associates), 5% (v/v) chick embryo extract (prepared from 10-d-old embryos as previously described⁶), 50 $\mu\text{g ml}^{-1}$ penicillin and 50 U ml^{-1} streptomycin. In our initial studies we used conditioned medium prepared by incubating standard culture medium with skeletal myotube or heart cell cultures for 72 h; the conditioned medium was passed through a Millipore filter before use.

When grown in conditioned medium, CG neurones rapidly extended processes and survived for many weeks in culture (Figs 1, 2a). Combining the cell counts for neurone cultures 1–3 weeks old yielded a value of $6,040 \pm 160$ (s.e.m. 52 cultures) for the mean number of surviving neurones per ganglion used to prepare the cultures. This represents 93% of the neurones previously shown to be present at the time of dissection³. Ganglionic non-neuronal cells seemed to be unnecessary for neuronal survival in these conditions, as few such cells were usually present (Fig. 1). Furthermore, 48-h exposure of the cultures to cytosine arabinoside, a drug that kills rapidly dividing cells such as ganglionic non-neuronal cells⁵, only slightly reduced neuronal survival (Table 1).

Assays of CAT activity indicated that the neurones continued to develop along cholinergic lines. CG neurones grown with and without myotubes developed comparable levels of CAT activity, and the levels increased about 10-fold between days 3 and 15 in culture (Fig. 2b). Lactate dehydrogenase (LDH), a cytoplasmic enzyme common to many cell types, was assayed as a marker for cell cytoplasm. LDH activity increased three- to fourfold over the same period. Thus, at least a part of the increase in CAT activity represented an increase in specific activity (CAT/LDH), suggesting that neuronal development as well as net growth had occurred.

Survival of CG neurones was promoted independently by factors contributed by embryo extract in the medium and by factors derived from heart cells during the conditioning period. Unconditioned medium containing embryo extract supported complete neuronal survival and the development of high levels of CAT activity (Table 1). Nearly complete survival was also achieved in heart-conditioned medium lacking embryo extract, although lower levels of CAT activity were observed in this case. In unconditioned medium lacking embryo extract, the neurones failed to extend processes and degenerated after 1–2 d. It remains to be determined how many factors are involved and whether the heart cell factors are the same as those found in

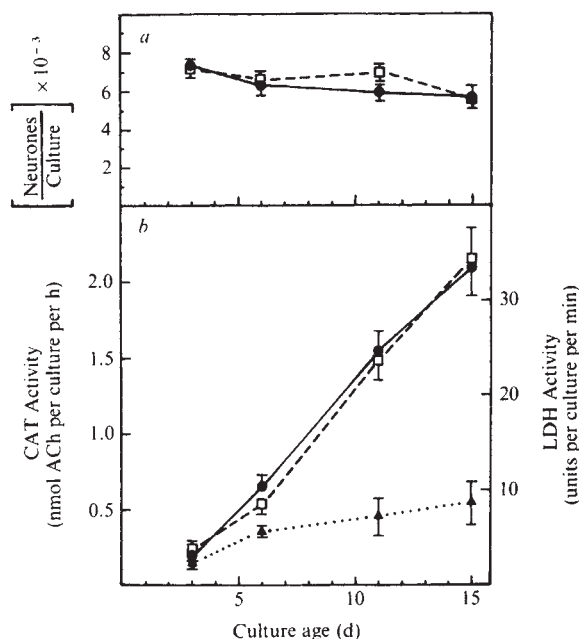


Fig. 2 Survival and development of CG neurones in culture. CG neurones from 8-d-old embryos were grown either with skeletal myotubes or alone with heart-conditioned medium. At the indicated times cultures were counted for neurones by examining 15 uniformly spaced fields of view at $\times 200$ with phase contrast optics. Neurones were evenly distributed over the culture surface and could be distinguished by morphological criteria⁴. Such cells were previously confirmed to be electrically excitable⁴ and could be autoradiographically labelled by high affinity ^3H -choline uptake¹³. ^3H -Thymidine autoradiography experiments demonstrated that CG neurones did not undergo cell division in culture (R.N. and D.K.B., unpublished observations). After counting the neurones, cultures were assayed for CAT and LDH activity as previously described¹⁴. Naphthyl vinyl pyridine (0.5 mM), a specific inhibitor of CAT, inhibited at least 80% of the activity. *a*, Neuronal survival; *b*, CAT and LDH activity. Neurones grown alone, $\square$; neurones grown with myotubes, $\bullet$; LDH activity for neurones grown alone, $\triangle$. Each point represents the mean $\pm$ s.e.m. for 7–10 cultures taken from 2–5 different cell platings. Background values for CAT and LDH activities were determined on culture wells treated identically to cell cultures except for the omission of cells; background values were always less than 10% of the culture values and were subtracted.

embryo extract. The differences in CAT activity per neurone observed for the various culture conditions (Table 1) raise the possibility that cholinergic development in the neurones may respond to different signals from those controlling neuronal survival. Other workers have described a factor in heart-conditioned F12 medium supplemented with 10% fetal calf serum that permitted neurite extension by CG neurones *in vitro*, but did not promote long-term survival in the conditions used^{6–8}.

These results demonstrate that neither nerve-muscle synapse formation nor direct contact with muscle cells is necessary for the survival and development of CG neurones if appropriate factors are supplied in the culture medium. (The role of possible nerve-nerve synapses in such conditions⁹ has not been examined.) Furthermore, it is clear that CG neurones are not irreversibly committed to cell death *in vivo*, even as late as the beginning of the normal death period. It might be argued that neurones in culture remain trapped at a developmental stage before that at which death occurs *in vivo*. We think that this is unlikely as the neurones innervated myotubes when present⁴ and they developed high levels of CAT activity with or without myotubes. CAT activity on a per neurone basis at 15 d in culture was about five times greater than that reported for the sum of activity for the CG plus iris (postsynaptic target) tissue for 8-d embryos¹⁰. If the *in vivo* values could be corrected for contributions for preganglionic terminals, the comparison would be even

more favourable. CAT levels did not increase as rapidly in culture, however, as they did *in vivo*.

These findings are consistent with the suggestion that survival of parasympathetic neurones *in vivo* may be regulated in part by availability of a factor supplied by postsynaptic target cells¹¹. Several lines of evidence support such a model for regulation of cell death for sympathetic neurones by nerve growth factor, although other interpretations of the data remain possible¹².

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GABA and glycine may share the same conductance channel on cultured mammalian neurones

ANALYSIS of conductance fluctuations induced during application of putative neurotransmitters to postsynaptic membranes has led to estimates of the unitary conductance and kinetics of the underlying ionic channels. Estimates have been made for a variety of receptor-coupled channels activated on invertebrate and vertebrate membranes (for review see ref. 1). Recently we have applied the technique to mouse spinal neurones grown in tissue culture and have estimated the conductance and duration of the single channel events underlying responses to the putative inhibitory transmitter γ -aminobutyric acid (GABA)². We have extended our studies to another inhibitory transmitter, glycine, and report here that channels activated by glycine have properties distinctly different from those activated by GABA and that the amino acid receptors may share a conductance mechanism.

Neurones were dissociated from the spinal cords of 13–14-d-old mouse embryos and grown in tissue culture³ until large enough to be penetrated with two microelectrodes (20–25 μ M cell bodies). Recordings were made on the modified stage of an inverted microscope using phase contrast optics at $\times 250$ magnification. GABA and glycine were applied to the cell body by microiontophoresis. $MgCl_2$ was added to a final concentration of 10 mM to suppress spontaneous synaptic activity and allow easier examination of the postsynaptic events. The temperature of the experiments was $26 \pm 1^\circ C$. When recording with either KAc or KCl microelectrodes, the inversion potential of the GABA and glycine responses are identical, averaging about -63 mV with KAc microelectrodes and about -20 mV with KCl microelectrodes⁴. This difference in inversion potential for the two recording situations is a result of increased intracellular Cl^- concentration created by Cl^- leakage from KCl

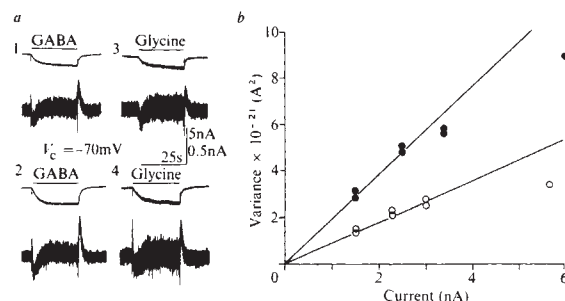


Fig. 1 GABA and glycine activate channels with different conductances on the same spinal neurone grown in cell culture. *a*, Membrane current recordings from a neurone voltage clamped to -70 mV show inward current responses during iontophoresis of GABA (1, 2) and glycine (3, 4). Iontophoresis duration is marked by bars above traces. Each pair of traces contains a d.c.-coupled trace above and a high-gain, a.c.-coupled trace below. Membrane current responses to GABA and glycine are associated with dose-dependent increases in the thickness of each trace, reflecting corresponding increases in conductance fluctuations. *b*, The variance in current fluctuation is directly related to the amplitude of the membrane current response to GABA (○) and glycine (●). Increases in variance obtained during the two sets of responses shown in *a* were computed along with those observed during two other amino acid responses and these plotted as a function of membrane current response. The first three points are approximated by straight lines whose slopes give estimates of single channel conductances of 16.4 pS and 36.4 pS for GABA and glycine, respectively. See text for explanation of deviations of the fourth points from the straight line.

microelectrodes. Prolonged amino acid responses frequently reverse polarity when recorded with KAc microelectrodes, presumably due to the time-dependent change in driving force induced by sustained activation of Cl^- conductance⁴. In contrast, KCl recordings permit a relatively more stable, albeit altered, driving force. For these reasons fluctuation experiments have been carried out using KCl microelectrodes.

Cells were voltage clamped so that the difference between the holding potential and the inversion potential for the amino acid responses (the driving force) was about 60 mV. Both amino acids caused a dose-dependent increase in inward current which was associated with a dose-dependent increase in the fluctuations of the current signal (Fig. 1). Amino acid-induced increase in variance was calculated by subtracting the baseline variance obtained before or after the iontophoretic application. This particular fluctuation analysis requires three basic assumptions: (1) the variance is derived from a statistical variation in the number of open ion channels activated by the amino acids about a mean level of open channels; (2) each ion channel event is rectangular and constant in amplitude; and (3) the probability of any channel being open is small¹. With these assumptions an estimate of the single channel conductance, γ , can be made from measurement of the agonist-induced variance, σ^2 , using the equation

$$\gamma = \sigma^2 / (\Delta I (V_c - E_r))$$

where ΔI is the agonist-induced membrane current, V_c is the potential to which the cell is voltage-clamped and E_r is the reversal potential of the current response. Glycine current responses were associated with a larger variance than GABA current responses of similar amplitude. The first three points of both series of amino acid responses have been approximated by straight lines whose slopes give γ values of 15 and 29 pS for GABA and glycine, respectively. The fourth points fall below the straight lines, indicating an apparent decrease in γ . Both of the largest current responses were partially desensitized. The apparent decrease may reflect a large increase in the probability of non-desensitized channels being open, contrary to one of the assumptions on which fluctuation analysis is predicted. Estimates of γ were made for GABA and glycine applied to the

same spinal neurones ($n=4$) and to different spinal neurones ($n=12$ cells for GABA and 6 cells for glycine). When the amino acids were compared on the same neurone τ estimates were (mean $\pm$ s.d.) 14.7 ± 3 and 30.4 ± 8.4 pS for GABA- (71 observations) and glycine-activated channels (50 observations), respectively. Estimates made on separate cells were 18 ± 8.2 pS for GABA (271 observations) and 30.4 ± 9.5 pS for glycine-activated channels (71 observations).

Channel duration estimates were made by performing spectral analyses of baseline and amino acid-induced fluctuations and then subtracting the former from the latter power density spectra. Power density spectra were calculated as the Fourier transform of at least 8,192 point samples of the fluctuations digitised at 1, 2 or 5 ms per point⁵. Spectra of GABA- and glycine-induced fluctuations could usually be approximated by a single lorentzian of the form $S(f)/S(0) = 1/[1 + (f/f_c)^2]$, where $S(f)$ is the spectral density as a function of frequency, f , and f_c is the frequency where the spectral density has decreased to half the value of the zero-frequency asymptote, $S(0)$, of the spectrum. Such an approximation is consistent with the hypothesis that the fluctuations arise from a single population of channels whose duration is exponentially distributed. The average channel duration, τ , compared on the same four neurones was (mean $\pm$ s.d.) 26.7 ± 7.7 ms (71 observations) and 4.8 ± 1.3 ms (50 observations) for GABA- and glycine-activated channels, respectively. Estimates of durations derived from amino acid responses obtained on different neurones were 20 ± 6.6 ms for GABA- (271 observations) and 5.2 ± 1.6 ms for glycine-activated channels (71 observations).

From these results it is clear that single channel properties activated by GABA and glycine are not identical, with GABA-induced events being lower in conductance and longer in duration than those induced by glycine. The differences in the properties of the single channel events may reflect differential binding of the amino acids to the same receptor-channel complex, as has been suggested to account for different single

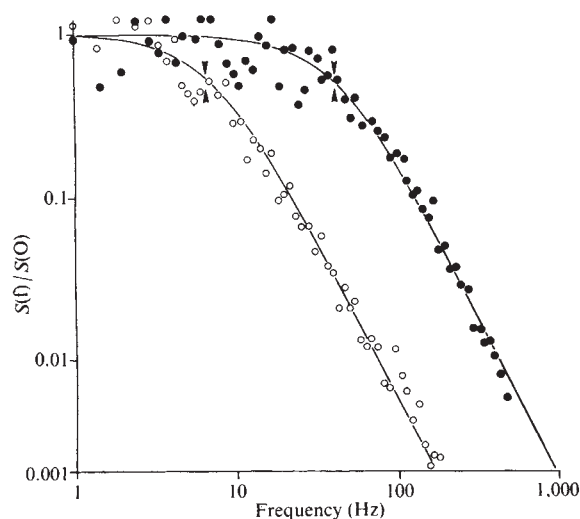


Fig. 2 Power density spectra of amino acid-induced current fluctuations show different durations for GABA- (○) and glycine- (●) activated channels. These spectra were calculated from results obtained on the same spinal neurone as in Fig. 1. The spectra shown are the differences between spectra derived from amino acid-induced fluctuations and those derived from baseline fluctuations. They have been normalised by dividing each spectral density point, $S(f)$, by the zero-frequency asymptote of the spectrum, $S(0)$. Points across frequency bands have been averaged to smooth the spectra. The points are approximated by Lorentzians (solid lines) of the form $S(f)/S(0) = 1/[1 + (f/f_c)^2]$. Double arrowheads indicate the half-power frequency, f_c which is related to mean channel duration, τ , by $\tau = 1/\pi f_c$. In this cell, f_c for GABA is 6.5 Hz and f_c for glycine is 41 Hz, corresponding to channel durations of 24.5 and 3.9 ms, respectively.

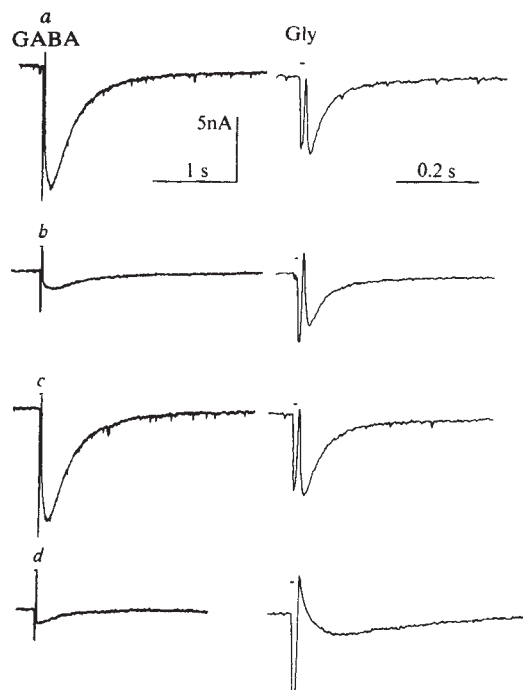


Fig. 3 GABA and glycine interact on cultured spinal neurones. The cell illustrated was voltage clamped to -80 mV and GABA and glycine iontophored either separately (*a*, control; *c*, recovery) or together (*b*, during prolonged iontophoresis of GABA; *d*, during prolonged iontophoresis of glycine). The amplitude of the GABA current response is markedly depressed and its time course slowed when superimposed on a prolonged, desensitising dose of GABA (*b*, left-hand trace). A depression of the glycine current response without apparent change in time course is also evident during prolonged GABA iontophoresis (*b*, right-hand trace). Conversely, the GABA response is markedly depressed without apparent change in time course during prolonged glycine iontophoresis (*d*, left-hand trace). The glycine response is also considerably depressed and its time course slowed during the prolonged, desensitising glycine application (*d*, right-hand trace).

channel events activated by various cholinergic agonists applied to endplate regions of muscle membranes⁶. However, a single receptor site for neutral amino acids on spinal neurones is not supported by several lines of investigation. Convulsant drugs can selectively antagonise the pharmacological responses of spinal and sensory neurones to either of the amino acids without affecting responses to the other⁷⁻¹⁰. The amino acids do not affect each other's affinity for receptor sites as reflected in binding studies on membranes extracted from the central nervous system (refs 11-13 and E. J. Peck, personal communication). Structure-activity studies with conformationally restricted GABA analogues have indicated an 'active conformation' of GABA which cannot be matched by any configuration of glycine¹⁴. Some membranes respond to GABA but not to glycine^{15,16}. We have also found a selective antagonism of the amino acid responses on cultured spinal neurones by specific convulsants (refs 17, 18 and unpublished observations) and a selective enhancement of GABA responses by barbiturates^{19,20} and benzodiazepines²¹. Cultured cells grown in the presence of GABA respond to glycine but not GABA, while cells grown in glycine respond to GABA, but not glycine²². These findings, coupled with the present results, suggest that the different single channel properties activated by GABA and glycine reflect engagement of physically distinct receptor-channel complexes on neuronal membranes.

This notion is not supported by observations made on amino acid current responses elicited on membranes acutely desensitised to one or the other amino acid. For example, sustained applications of GABA induced a current and conductance response which rapidly desensitised. Current and conductance

responses to glycine iontophoresed onto membranes desensitised to GABA were depressed in a reversible manner relative to those obtained in the absence of GABA, while the time course of the responses did not seem to be altered (Fig. 3a-c). (The time course of desensitised GABA and glycine responses was always slower than those obtained in control.) Conversely, sustained application of glycine sufficient to induce desensitisation of glycine responses reversibly depressed the current and conductance response to GABA relative to control with little change in its time course (Fig. 3d). Similar depressions of one amino acid response during sustained iontophoresis of the other were observed in each of 11 spinal cord cells where the interaction was examined. The depressions of the amino acid responses were not associated with a change in the driving force for the amino acid responses (unpublished observations).

One explanation for these results is that both amino acids can compete for each receptor site but only activate conductance at the appropriate receptor. The inappropriate amino acid would thus interfere with channel activation by the appropriate amino acid at the receptor. However, a variety of studies have failed to show an interaction between the amino acids (refs 11-13, 22, E. J. Peck, personal communication) and we have found that GABA responses elicited on cultured dorsal root ganglion cells are not altered by sustained application of glycine, which does not affect the membrane properties of the cells (unpublished observations). Thus, it seems unlikely that the interaction takes place at the receptor level. Alternatively, GABA and glycine receptors may share a common channel which can still be activated by glycine when GABA receptors are largely desensitised but which is difficult to activate by GABA when glycine receptors are only partially desensitised and thus almost continuously using these channels. This latter explanation suggests that postsynaptic Cl^- conductance mechanisms might be coupled to several amino acid receptors and that the properties of the resulting single channel event are determined by the type of amino acid released from presynaptic terminals.

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Acetylcholine receptors in organ-cultured human muscle fibres

ORGAN culture of nerve and muscle facilitates investigations which cannot easily be carried out *in situ*^{1,2}. We have developed a method for maintaining human muscle in organ culture which should allow us to investigate normal as well as various pathological properties of muscle fibres. We report here the use of these preparations to compare junctional and extrajunctional channels, induced by acetylcholine (ACh), in human muscle. In addition we have made a preliminary study of miniature end-plate currents, in normal and myasthenia gravis endplates, following organ culture.

Human intercostal nerve-muscle obtained from myasthenia gravis and control patients was dissected in sterile conditions to give small bundles of fibres (~100). Fibre bundles were pinned through their tendons and mounted, under slight tension, on Sylgard frames. They were maintained in Trowell's T-8 culture medium (Gibco) or Dulbecco's minimal essential medium (Gibco) containing 100 IU ml⁻¹ penicillin and streptomycin and 0.02 mM glutamine in an atmosphere of 95% O₂/5% CO₂ (ref. 3). Cultured muscle fibres had 'overshooting' action potentials and resting potentials of over 70 mV, and remained viable for periods of at least 2 weeks at a constant temperature of 23 °C. At this temperature the rate of metabolism is greatly reduced⁴. Electrophysiological methods were as previously described⁵.

Extrajunctional ACh sensitivity was studied at the muscle-tendon junction, where it is known to occur in other species^{6,7}. A voltage recording intracellular microelectrode was inserted near the tendon to measure the response to a 1-2 nC ionophoretic pulse of ACh applied to the surface of the fibre. The ACh pipette was moved in steps of 50-100 µm (Fig. 1). Some fibres (approximately 5-50%) of normal human muscle were found to have a marked ACh sensitivity at the tendon junction. The sensitive area covered a length of ~800 µm, that is, a small fraction of the total fibre length of approximately 1-2 cm. The peak of sensitivity sometimes occurred several hundred µm away from the

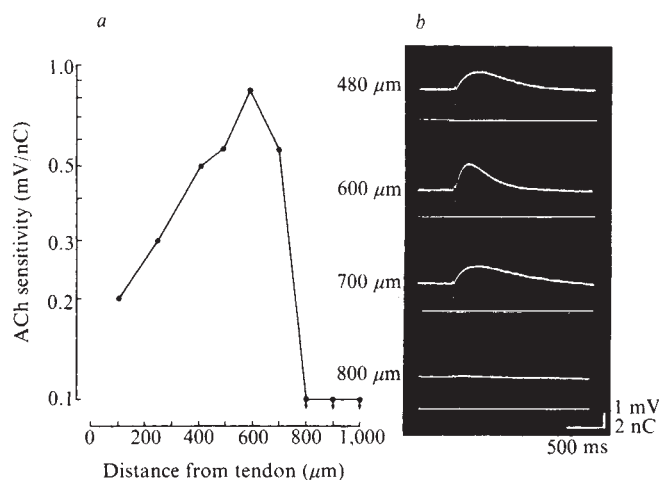


Fig. 1 *a*, Distribution of extrajunctional acetylcholine sensitivity, at the muscle-tendon junction of a fresh normal human muscle fibre, measured in response to 2 nC pulses of ACh applied from a micropipette at various distances from the tendon. Note that the peak of sensitivity in this fibre apparently occurs at 600 µm from the tendon although the presence of connective tissue in the tendon region makes the actual site of peak sensitivity uncertain. At 800 µm or more from the tendon, sensitivity was undetectable. *b*, Examples of the potential changes produced by ACh applied to the tendon region (at 480, 600, 700 and 800 µm from the tendon); same experiment, as shown in graph. The drug ejection currents passing through the micropipette are monitored on the lower trace in each pair. Calibration 1 mV or 2 nC, 500 ms.

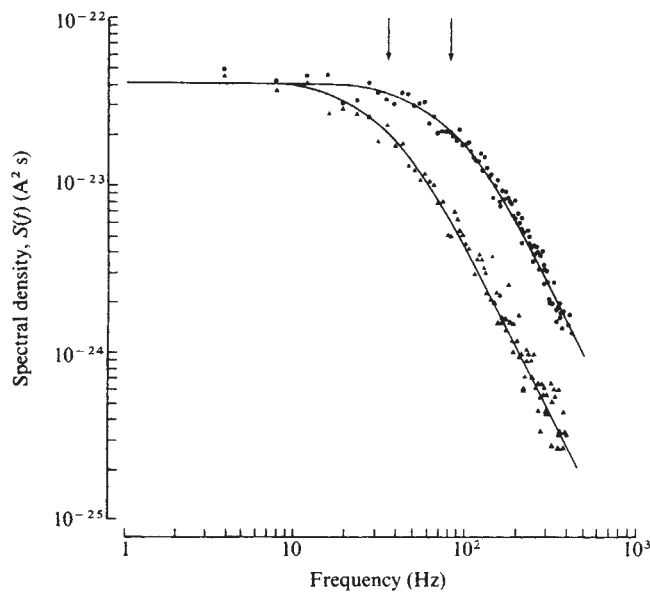


Fig. 2 Power spectra of current noise produced by steady ionophoretic application of acetylcholine to a voltage clamped endplate (●) and an extrajunctional site at the muscle-tendon junction (▲) in human muscle cultured at 23 °C for 5 d. The spectra are from two fibres, in the same bundle, at clamp holding potential $V_m = -80$ mV, temperature, 21 °C. Endplate spectrum (●) was obtained from 136 averaged spectra (four noise runs). Half-power frequency indicated by arrow is $f_c = 87$ Hz, hence $\tau_{\text{noise}} = 1.8$ ms. Mean current, $\mu_I = 19.7$ nA and the single channel conductance calculated from the fitted Lorentzian curve is, $\gamma = 25.14$ pS. Extrajunctional spectrum (▲) was obtained from 76 averaged spectra (three noise runs). Half-power frequency indicated by arrow is $f_c = 35$ Hz, hence $\tau_{\text{noise}} = 4.5$ ms. Mean current, $\mu_I = 4.8$ nA and the single channel conductance calculated from the fitted curve is, $\gamma = 11.75$ pS. Vertical axis applies to the extrajunctional spectrum; multiplication of the ordinate values by 3.03 gives the spectral density values ($\text{A}^2 \text{s}$) for the endplate noise. After feeding into a 1,000-Hz active ($1/f^8$) low pass filter, data was digitalized at 2,000-Hz sample rate and edited to remove spontaneous, miniatures and artefacts. Control noise, taken immediately before application of ACh, has been subtracted. Fast Fourier transform was calculated for 512 point segments. The single channel conductance was normally estimated from both the variance, σ^2 , of the current noise and the zero frequency asymptote, $S(0)$, of the spectrum; $\gamma = \sigma^2 / \mu_I (V_m - V_{\text{eq}})$ and $\gamma = S(0) f_c / \mu_I (V_m - V_{\text{eq}})$ where the equilibrium potential, V_{eq} , for ACh was taken as zero mV.

tendon (Fig. 1). Normally, sensitivity was not detectable between the tendon junction and the endplate. Fibres cultured for up to 14 d did not acquire sensitivity in this region. Also, in only one out of eight cultures kept at 36 °C for up to 14 d did we find evidence for denervation supersensitivity. This is in sharp contrast to what has been found in rat muscles which rapidly acquire supersensitivity in the whole fibre after denervation in similar culture conditions².

Acetylcholine sensitivity was not detected in the muscle tendon region of any of the myasthenic muscles studied (16 fibres, 3 muscles). This is probably related to the same process which causes reduced ACh sensitivity of endplates in myasthenic fibres.

Properties of junctional and extrajunctional channels were compared in normal human muscle cultured at 23 °C by analysing the voltage clamped noise⁸⁻¹⁰ which occurred during steady ionophoretic application of ACh to a junctional site or to a tendon region. Power density spectra of current fluctuations are shown in Fig. 2. In most cases spectra were fitted with a single Lorentzian component ($1/\text{frequency}^2$), and the frequency, f_c , at which the power is reduced to one half of its maximum value, gave the mean life-time of the open channel, τ_{noise} , according to $\tau_{\text{noise}} = 1/(2\pi f_c)$. Power spectra of junctional and extrajunctional noise are similar in form (Fig. 2) but their half-power frequencies differ revealing that the open-state of

extrajunctional channels is prolonged when compared with that of endplate channels.

In the example shown in Fig. 2 extrajunctional channels have a mean life-time of 4.5 ms compared with 1.8 ms for the junctional channel from the same muscle. The mean open-time of junctional channels in culture was $\tau_{\text{noise}} (\text{junctional}) = 1.55 \pm 0.08$ ms ($n = 7$ fibres); the comparable value for extrajunctional channels was $\tau_{\text{noise}} (\text{extrajunctional}) = 3.52 \pm 0.27$ ms ($n = 5$ fibres), $V_m = -80$ mV, temperature, 21 °C. The single channel conductance, γ , derived from both the variance of the current noise and the zero frequency asymptote of the spectra, was in the range 20–25 pS for endplate channels and 11–16 pS for extrajunctional channels. The values of these channel parameters are similar to those obtained in the same muscles before culturing (see also ref. 11).

In both normal and myasthenic endplates miniature endplate currents (m.e.p.cs) are present for 5–7 d in muscles cultured at 23 °C indicating the time required for denervation of the endplates. It was therefore possible to assess the sensitivity of fresh and cultured endplates by comparing their m.e.p.c. amplitudes. Myasthenic m.e.p.cs are small apparently due to binding of

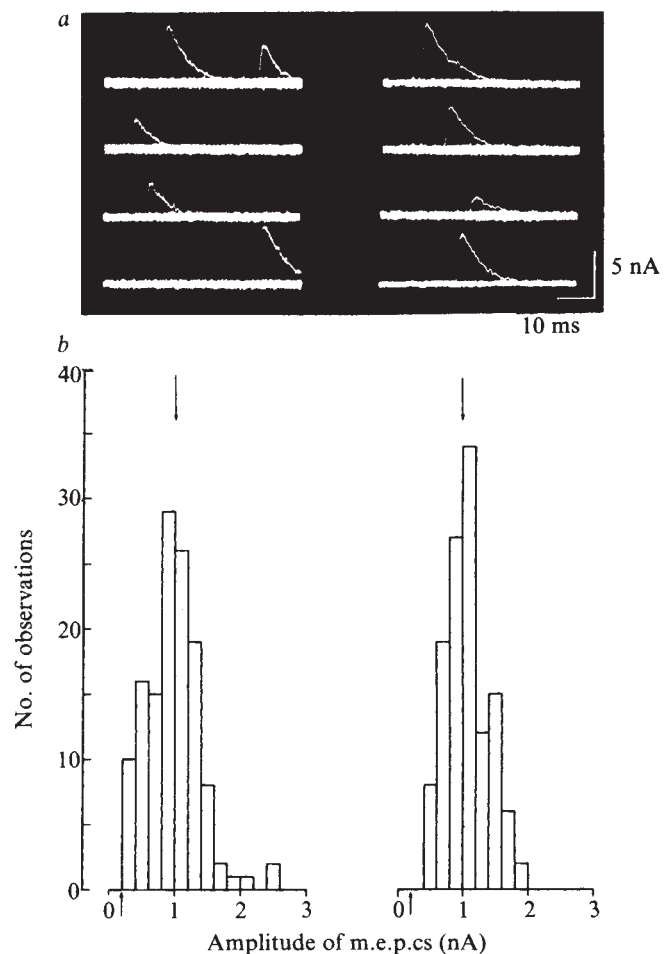


Fig. 3 *a*, Examples of miniature endplate currents at a fresh normal human endplate (left) and at an endplate from the same muscle after 5 d in culture (right). m.e.p.cs were recorded under voltage clamp ($V_m = -80$ mV, temperature, 21 °C) in the presence of prostigmine 2×10^{-7} g ml⁻¹ through a 500-Hz low pass filter which prolongs the rising phase and slightly attenuates the amplitude. Calibration, 5 nA and 10 ms. *b*, Amplitude distributions of m.e.p.cs at a voltage clamped myasthenic human endplate in fresh muscle (left) and at an endplate from the same muscle after 6 d in culture (right). Mean m.e.p.c. amplitude (arrows) at the fresh endplate = 0.99 ± 0.06 nA (mean $\pm$ s.e.m.); mean m.e.p.c. amplitude at an endplate after 6 d culture = 1.06 ± 0.03 nA. m.e.p.cs were obtained in the presence of prostigmine 2×10^{-7} g ml⁻¹ ($V_m = -80$ mV, temperature, 21 °C). Background noise level is indicated by arrows on abscissa.

anti-receptor antibodies to the ACh-receptor¹², which may cause a loss of functional receptors^{13,14}, and also a reduction in their number through an increased rate of degradation¹⁵⁻¹⁷. As circulating anti-receptor antibody occurs in the plasma of myasthenic patients¹⁸, the possibility arises that in its absence the m.e.p.cs would increase in amplitude if antibody could detach from receptors or if new receptors were incorporated into the membrane; although the latter process should be far from optimal in culture conditions at 23 °C.

The amplitude of m.e.p.cs at normal human endplates is not markedly changed after 5-6 d in our culture conditions. Figure 3a shows examples of m.e.p.cs recorded from a voltage-clamped normal endplate at zero culture time and from another endplate in the same muscle 5 d later ($V_m = -80$ mV, temperature, 21 °C, prostigmine 2×10^{-7} g ml⁻¹). In cultures of three myasthenic muscles there was no marked change in m.e.p.c. amplitudes after 5-7 d. Amplitude distributions of myasthenic m.e.p.cs from one endplate before culture and from another endplate in the same muscle 6 d later are shown in Fig. 3b.

We conclude that extrajunctional ACh receptors are normally present in some adult human muscle fibres and that the channels they operate differ from endplate channels in their kinetic properties. This is analogous to the situation in denervated frog^{8,19}, rat and mouse²⁰ muscle where properties of junctional and extrajunctional channels show differences comparable to those described here. However, the single channel life-time and conductance of the extrajunctional channels in adult human muscle are smaller than those in cultured human myotubes²¹. This dissimilarity as well as that between junctional and extrajunctional receptors in adult fibres may reflect differences in the receptor molecules themselves or in their membrane environment.

Finally our experiments indicate that if some of the reduced ACh sensitivity of myasthenia gravis endplates results from binding of an anti-receptor antibody, as has been suggested, then the antibody-receptor combination is practically irreversible when the muscle is maintained at 23 °C or the antibodies may unbind but leave the receptors still in a non-functional state.

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Is the red cell calcium pump regulated by ATP?

THE very low physiological level of calcium in the human red cell is maintained by a powerful ATP-fuelled Ca extrusion pump which has been extensively studied¹. Recently evidence has been accumulating that the kinetic properties of the active Ca flux and associated (Ca + Mg)ATPase activity depend very much on the experimental conditions and/or the manner of preparation of the red cell membranes or resealed ghosts²⁻⁴. The variability may reflect the nature or degree of interaction between the membrane Ca pump and a cytoplasmic activator protein described recently⁵⁻⁷, which could be involved in regulation of physiological Ca levels. In previous work with whole red cells^{8,9} or resealed ghosts¹⁰ the concentrations of the major Ca pump ligands, ATP and/or Ca, have been changing during the course of the reaction. Because of the possible importance of the ligand conditions in assessing the physiological functioning of the Ca pump, we have looked at (Ca + Mg)-dependent ATP hydrolysis in resealed ghosts with buffered ATP and Ca levels and at the active Ca flux with a buffered ATP level. The experiments reported here show that both the ATP hydrolysis and the Ca flux are activated with two distinct ATP affinities and this raises the possibility of regulation of the Ca pump by ATP in the region of the lower affinity.

For the ATPase measurements, the internal ATP concentration was clamped at a fixed level by incorporating [γ -³²P]ATP and a regenerating system into the ghosts. The regenerating system consisted of creatine kinase and creatine phosphate in chemical and isotopic equilibrium with the ATP. The method (details of which are given in the legend to Fig. 1) was developed previously¹¹ and has the advantage of allowing measurement of ATP hydrolysis at very low concentrations. The free Ca levels in the cells were maintained in the range 10^{-8} to 10^{-4} M by incorporating Ca buffers into the cells and equilibrating the Ca concentrations across the membranes by means of the Ca-ionophore A23817. Figure 1 shows a time course of ³²Pi release in cells prepared to contain 50 μ M ATP and about 6 μ M free Ca²⁺. Measurements of the internal Ca by flame photometry showed that it was approximately equal to the external Ca concentration at the beginning of the test incubation and remained constant (not shown). The rate of ³²Pi release was constant for about 15 min during which time the total ATP content of the ghosts must have turned over about 40 times. Linearity has been observed regularly at 1-1,000 μ M ATP demonstrating effective regeneration of ATP even at the high rates of ATP hydrolysis observed.

In several experiments in which ghosts were prepared to contain different Ca-buffer mixtures, and either 10 or 500 μ M ATP, the free Ca²⁺ concentration which sustained the half-maximal ATPase activity was about 0.2 μ M, that is, a 'high affinity' (see refs 3, 4).

Figure 2 shows the rate of (Ca + Mg)-dependent ATP hydrolysis as a function of the total ATP concentration in cells, with an optimal but relatively low free Ca²⁺ concentration of 6.3 μ M. The curve is clearly biphasic. The kinetic parameters calculated from the curve show a high affinity component with K_m 1-2 μ M and a V_{max} of about 4.7 mmol per l cells per h, and the low affinity region has a K_m of about 180 μ M with an overall maximal rate of about 16 mmol per l cells per h. The rate of ATP hydrolysis in resealed ghosts without Ca, that is Mg-dependent ATPase, was subtracted from the total activity with Ca and Mg at all ATP concentrations. The rate of Mg-ATPase did not vary between 10 and 500 μ M ATP and the measured value of 0.8 mmol per l cells per h was used to make the correction. The rate of (Ca + Mg)ATPase is presumably somewhat underestimated at very low ATP concentrations (< 10 μ M) but this does not alter the activation pattern.

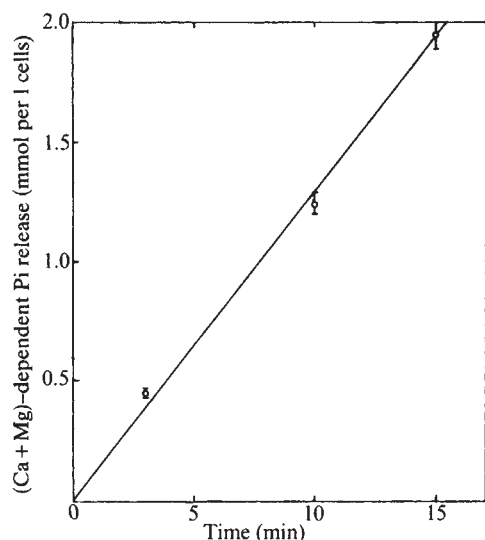


Fig. 1 (Ca + Mg)-dependent ATP hydrolysis in resealed red cells containing buffered concentrations of Ca^{2+} ions and ATP. See ref. 11 for details. Preparation of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and ^{32}P -creatine phosphate in chemical and isotopic equilibrium: 250 μM of ATP (containing $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ 1×10^6 c.p.m.) was added to 1 ml of a solution containing 50 mM creatine phosphate, 0.5 mM creatine, 12.5 mM MgCl_2 , 25 mM inosine, 0.5 mM ouabain, 50 mM HEPES (Tris) (pH 7.4 at 22°C) and 25 units of creatine kinase and hexokinase. After 1 h incubation at 37°C, 5 μmol of HEDTA (pH 7.4 at 22°C) was added, the solution was diluted 5 times and kept at 0°C. Preparation of resealed ghosts and measurement of ATP hydrolysis: red blood cells were washed 3 times with a standard 'wash solution' which contained: 130 mM NaCl, 10 mM KCl, 2.5 mM MgCl_2 and 10 mM HEPES (Tris) (pH 7.4 at 22°C). The cellular ATP was depleted by incubating the cells for 60 min at 37°C in a medium containing 6 mM inosine and 6 mM iodoacetamide. The cells were then washed 3 times at room temperature to remove inosine and iodoacetamide and packed at 1,500g for 10 min. Packed cells were lysed in 9 times their own volume of the diluted ice-cold solution containing the ATP regeneration system. After 2 min 2 M NaCl was added to restore the tonicity to 310 ideal milliosmolar, and the ghosts were incubated at 37°C for 10 min. The resealed ghosts were then washed 3 times, the first and third time with ice-cold medium and the second wash at room temperature. The final intracellular concentrations were: NaCl, 130 mM; MgCl_2 , 2.5 mM; HEDTA, 1 mM; ATP (+ $[\gamma\text{-}^{32}\text{P}]\text{ATP}$), 50 μM ; creatine phosphate, 10 mM; HEPES (Tris), 10 mM pH 7.4; creatine, 100 μM ; ouabain 0.1 mM; creatine kinase 5 units per ml and hexokinase 5 units per ml. The ghosts were packed at 25,000g for 1 min, suspended to 3–5% haematocrit in ice-cold wash solution containing 0.1 mM ouabain, and CaCl_2 0.71 mM plus HEDTA 1 mM, calculated to give 6.3 μM free Ca^{2+} (see ref. 12). Ca ionophore (A23187) was then added to a final concentration of 10 μM and the ghosts were incubated for 20 min at 0°C and for 5 min at 37°C before the measurements were begun. At various times at 37°C the ATP hydrolysis was terminated by the addition of glucose to a final concentration of 15 mM and further 10 min incubation at 37°C (this converts the remaining creatine phosphate and ATP to glucose-6-phosphate). The membranes were precipitated by 5% perchloric acid. 1 ml of membrane free extract was shaken for 30 s with 4 ml 0.5% ammonium molybdate in 5% (w/v) perchloric acid and 2 ml isobutanol, and the tubes were centrifuged at 1,000g for 30 s. Measured portions of the isobutanol layer were transferred to vials containing 0.5 ml of 2 M Tris solution and 8.5 ml Bray's scintillation fluid, and counted. The total ^{32}P counts were estimated by boiling 1 ml of the ghost suspension with 1 M HCl for 15 min and extracting ^{32}P as above. The rate of ATP hydrolysis is calculated from the fraction of breakdown of total phosphate substrate (creatine phosphate + ATP) per given time. Each point represents the average value ± 1 s.e.m. from tubes incubated in triplicate.

Experience showed that to observe biphasic curves the free Ca^{2+} within the cell must be less than about 1 mM. At higher concentrations (3–6 mM) we have observed an apparent hyperbolic saturation by ATP with a K_m of 5–10 μM and a severely depressed maximal rate of 3–6 mmol per l cells per h. The difference in the observed type of behaviour at low and high Ca is attributable to this inhibitory effect of high Ca concentrations, which is much more effective at the higher ATP concentrations than in the low ATP concentration range (not shown).

The curve in Fig. 2 could indicate that ATP combines twice with the Ca pump per turnover but it might also be explained by the action of two different (Ca + Mg)-dependent enzymes with only one involved in Ca transport. It was therefore also important to look at the ATP dependence of the active Ca flux. For the flux experiment of Fig. 3 the ATP levels were buffered again by the use of the creatine phosphate-creatine kinase system and the cells were loaded with 2.5 mM Ca (+ ^{45}Ca). Here, of course it is not possible to use the ionophore and so the internal Ca concentration drops during the course of the incubation. The cells in this experiment contained less than 1 mM total Ca immediately before the test incubation. The rate of ^{45}Ca efflux was constant for 5–15 min at the different ATP concentrations. A biphasic behaviour is clearly observed. The kinetic parameters for the two regions are respectively $K_m \approx 2 \mu\text{M}$, $V_{\max}$, 2.2 mmol per l cells per h and $K_m \approx 190 \mu\text{M}$ with an overall $V_{\max}$ of 7.3 mmol per l cells per h. Again if much higher initial Ca concentrations were used the biphasic pattern could not be clearly distinguished. In Fig. 3 the rates of Ca efflux are appreciably lower than the ATPase measured with low free Ca^{2+} concentration plus ionophore (see Fig. 2). This is due to a significant inhibitory effect of Ca even at the 0.5–1 mM level present in the flux experiment. The Ca inhibition seems to affect the $V_{\max}$, or turnover rate of the Ca pump without altering the apparent affinities or K_m for ATP. In parallel measurements of ATP hydrolysis estimated as in Fig. 2 but without ionophore, and Ca flux, made with ghosts containing 0.5–1 mM Ca^{2+} and 10 μM or 500 μM ATP, we have observed a ratio of Ca efflux to (Ca + Mg)-dependent ATP hydrolysis of 0.93 and 1.08 at the two ATP concentrations. This confirms the figure reported originally by Schatzmann¹⁰.

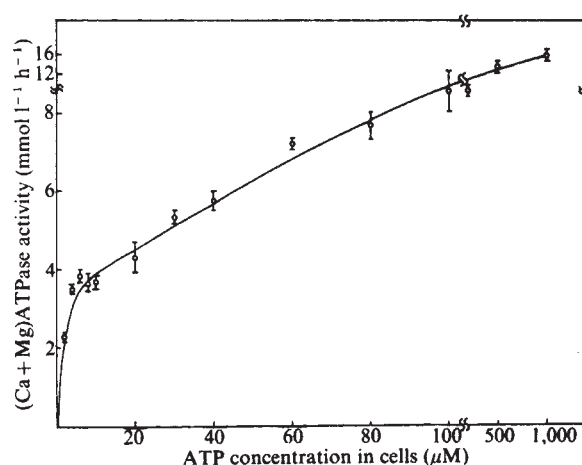


Fig. 2 ATP dependence of the (Ca + Mg)ATPase in resealed ghosts. Ghosts were prepared essentially as described in the legend to Fig. 1 except that: (1) the cells had been starved for 24 h at 37°C in the glucose free wash solution containing also 0.2 mM EGTA with no added Ca, and chloramphenicol (25 $\mu\text{g ml}^{-1}$), before a 2-h incubation with inosine and iodoacetamide; and (2) the lysing solution contained the appropriate concentrations of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, together with the ATP regeneration system. For measurement of (Ca + Mg)ATP hydrolysis the cells were incubated at 37°C for 15–60 min according to their ATP concentration. The rate of Mg-dependent ATP hydrolysis was measured over a 120 min incubation period in cells containing 10 μM or 500 μM ATP, and 1 mM EGTA.

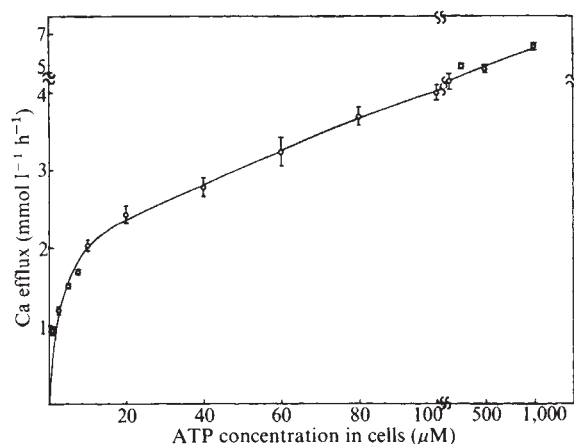


Fig. 3 ATP dependence of active Ca efflux from resealed ghosts. Red cells were washed as in the legend to Fig. 1 and starved as described in the legend to Fig. 2. 0.5 ml of packed starved red cells was lysed in 4.5 ml of ice-cold lysing solution containing: ATP, 1–1,000 μ M as indicated, creatine phosphate, 10 mM creatine kinase 25 units. CaCl_2 (containing ^{45}Ca 2×10^6 c.p.m.) 2.5 mM; HEDTA, 1 mM; MgCl_2 , 2.5 mM; HEPES (Tris), 10 mM (pH 7.4 at 22 °C); inosine, 5 mM; and ouabain 0.1 mM. After 2 min 2 M NaCl was added to restore tonicity to 310 ideal milliosmolar. The cells were incubated for 10 min at 37 °C and washed as in the legend to Fig. 1 (EGTA, 0.5 mM was added to the warm wash solution). The cells were then suspended at 2–3% haematocrit in ice-cold wash solution. The Ca flux was initiated by transferring the tubes to a 37 °C water bath. At various times the efflux was terminated by removal of 0.5 ml of the suspension into 0.7 ml of ice-cold wash solution containing 170 μ M LaCl_3 . The cells were spun down for 2 min at 12,000g in an Eppendorf Microcentrifuge, and 1 ml supernatant was transferred into vials containing 9 ml of Brays scintillation fluid, and counted. Radioactivity in the supernatant at zero time was subtracted from that at all other incubation times. The total ^{45}Ca counts present initially in the cells was obtained by allowing complete efflux in a 2-h incubation at 37 °C. The concentration of Ca in the cells before the 37 °C incubation was estimated either from the total radioactivity per known volume of cells and the specific activity of the ^{45}Ca in the lysing solution, or by flame photometry. For the latter measurement 50 μ l of ghosts of known haematocrit (25–50%) were lysed in 1 ml of perchloric acid 1.5% w/v. The precipitated protein was spun down and the supernatant Ca was read against standard CaCl_2 solutions, 25–100 μ M made up in 1.5% PCA and the appropriate volume of wash solution (containing NaCl) added with the cell suspension. Both methods give similar results. In the experiment of Fig. 3 the initial Ca concentrations were 0.8–1.1 mM. The rate is calculated from the fractional efflux of the total Ca per given time. Each point represents the average value ± 1 s.e.m. from tubes incubated in triplicate.

The biphasic ATP activation curve and the constancy of the ratio of Ca flux to ATP hydrolysis at 10 and 500 μ M ATP suggest strongly that the ATP hydrolysis at both high and low ATP levels (Fig. 2) is involved in the working of the Ca pump.

In previous observations of ATP activation of the ATPase, using broken membranes somewhat conflicting results have been reported. Wolf¹³ and Schatzmann¹⁴ observed an apparent simple saturation behaviour with K_m values for Mg-ATP in the range 20–50 μ M. Wolf *et al.*¹⁵, working with a partially purified (Ca + Mg)ATPase, and Sharff and Foder¹⁶, have reported Hill coefficients of < 1 for activation by ATP but no evidence for a very high affinity activation as in the present resealed ghost experiments. On the other hand Sarkadi *et al.*¹⁷, looking at the active Ca flux in inside-out vesicles have seen a high ATP affinity. It seems possible that the differences between the resealed ghosts and broken membranes reflect changes that occur during the membrane preparations or result from the inhibitory effects of high Ca^{2+} mentioned above.

The reaction mechanism of the Ca pump-associated (Ca + Mg)ATPase involves a phosphorylated intermediate¹⁸. Rega

and Garrahan¹⁹ have shown that formation of the phosphoenzyme requires ATP at 1–2 μ M, that is, in the high affinity range. Thus it seems possible that the increase in the turnover rate at the higher ATP concentrations is a regulatory function of the nucleotide acting in a non-phosphorylating role. The cell membrane (Na + K)ATPase is phosphorylated by ATP acting at a high affinity site and in addition an extra non-phosphorylating function of the ATP at a low affinity site is well documented for both kidney membranes²⁰ and resealed red cells where we previously reported biphasic ATP activation curves¹¹. The non-phosphorylating function of ATP involves regulation of the rate of a conformational transition, which is probably involved in the transport of K^+ ions^{20–22}.

As the membrane Ca pump and Na pump show a number of similarities in the reaction mechanism it would be interesting if regulation of transport linked conformational changes by ATP is found to be another common feature of the active cation pumps.

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Physiological correlation between lactate dehydrogenase genotype and haemoglobin function in killifish

ERYTHROCYTE organic phosphate concentration is known to be an heritable trait in both humans and rats^{1–3}. In humans this trait has been correlated with genetic variability of both pyruvate kinase and hexokinase². However, similar phenomena have not been reported in lower vertebrates. During our studies on the allosteric regulation of fish haemoglobins by ATP (ref. 4) we observed significant variability in red cell ATP concentrations among individuals of the killifish *Fundulus heteroclitus*, and wondered whether this variability in organic phosphate could be the result of a genetic component. Consequently, we analysed *F. heteroclitus* for red cell ATP, concomitantly screening for enzyme variants. We report here that not only are ATP levels under genetic control, but they are also highly correlated with lactate dehydrogenase (LDH) phenotype. To our knowledge, this is the first such correlation reported. Moreover, as ATP is the major allosteric modifier of *F. heteroclitus* haemoglobin-oxygen affinity^{5,6}, this study provides a basis for an association

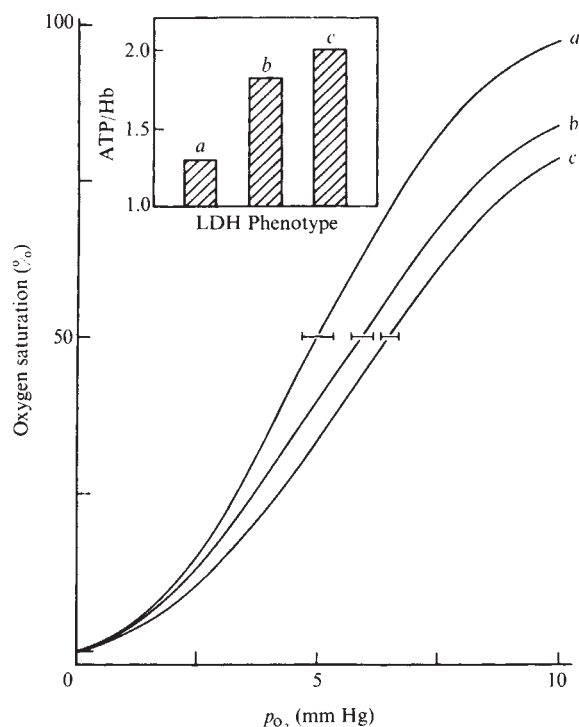


Fig. 1 Oxygen equilibria curves for whole blood from *F. heteroclitus* determined with an Aminco oxygen dissociation analyser. The ordinate is the fractional saturation of haemoglobin by oxygen and the abscissa the partial pressure of oxygen (p_{O_2}) in mm Hg. The standard errors of the mean p_{O_2} ($n=6$) for 50% saturation are represented by horizontal bars. Intra-erythrocyte ATP/Hb ratios for the three phenotypes are presented in the inset. ATP was determined by the firefly luciferase assay¹¹. *Fundulus* haemoglobin concentrations were determined as described previously⁵. The values for ATP/Hb are: a, $LDH-B^aB^a = 1.27 \pm 0.08$ ($n=6$); b, $LDH-B^aB^b = 1.83 \pm 0.11$ ($n=5$); c, $LDH-B^bB^b = 2.00 \pm 0.06$ ($n=5$). All animals were acclimated to laboratory conditions for 30 d before assays. Temperature, 20.5°C, pH 7.4.

between LDH and the fish's external environment through the physiological role of haemoglobin at the organism-environment interface.

There are three major electrophoretically distinguishable phenotypes of the heart-type LDH (the *LDH-B* locus) in *F. heteroclitus*^{7,8}. The polymorphism is due to two co-dominant alleles which exhibit a dramatic north-south cline in gene frequency along the Atlantic coast of the US^{9,10}. The two common *LDH-B* homozygous phenotypes yield fast ($LDH-B^aB^a$) and slow ($LDH-B^bB^b$) bands on electrophoresis, whereas the heterozygote phenotype ($LDH-B^aB^b$) gives the classic five-banded pattern⁸. The *B* locus is the only LDH expressed in the red blood cells of this fish⁸. When intra-erythrocyte ATP levels (expressed as molar ratios of ATP/Hb) are compared for individuals of different *LDH-B* phenotype, a significant association ($F_{[2,13]} = 20.58$, $P < 0.001$; index of association $\omega^2 = 0.76$) is found (see inset in Fig. 1).

ATP is the major organic phosphate in the red blood cells of *F. heteroclitus*⁵. Its role as an allosteric modifier of fish haemoglobins is similar to that of 2,3-diphosphoglycerate in mammalian red blood cells, in that it decreases the affinity of haemoglobin for oxygen⁵. A decrease in haemoglobin-oxygen affinity is characterised by an increase in the partial pressure of oxygen at which one-half of the haemoglobin is saturated with oxygen (the P_{50}). As the intra-erythrocyte ATP/Hb ratio is different for each of the *LDH-B* phenotypes, we expected to find differences in haemoglobin-oxygen affinity also. In agreement with this expectation (Fig. 1), $LDH-B^aB^a$ homozygotes with the lowest ATP/Hb ratio have the highest oxygen affinity and $LDH-B^bB^b$ homozygotes with the highest ATP/Hb value have the lowest oxygen affinity. At the same partial pressure ($p_{O_2} = 10$ mm Hg)

the haemoglobin from $LDH-B^aB^a$ fish is 96% saturated with oxygen, whereas that of $LDH-B^bB^b$ individuals is only 80% saturated. Such differences in oxygen loading would be exaggerated *in vivo* when blood pH is reduced following an increase in temperature and/or anaerobic metabolism⁵.

Our work on the haemoglobin components of *F. heteroclitus* indicates that the shift in oxygen equilibria does not result from a difference in the distribution of the haemoglobin components between the *LDH-B* phenotypes, as all individuals express the same complement of components. In addition, each iso-haemoglobin responds similarly to the changes in pH, temperature and ATP concentration⁵. Therefore, the differences in intra-erythrocyte ATP concentrations reported here must be either the result of: (1) functional differences between the *LDH-B* allozymes which affect cellular ATP levels, or (2) the regulation of ATP levels by other enzymes which are genetically linked to the *LDH-B* locus. Studies are now underway to answer this question. Also, selection experiments are in progress to determine the feasibility of oxygen and pH as selective agents for the *LDH-B* phenotypes of *F. heteroclitus*.

Whatever the molecular basis for these ATP differences, it is clear that the $LDH-B^aB^a$ phenotype has a lower intra-erythrocyte ATP concentration than $LDH-B^bB^b$ individuals and as a consequence, a higher haemoglobin-oxygen affinity (Fig. 1). Fish with the $LDH-B^aB^a$ phenotype might therefore have a selective advantage when competing with individuals of the other two phenotypes in a low oxygen environment. Final resolution of this phenomenon may help resolve the controversy between exponents of the 'selectionist' and 'neutralist' evolutionary theories concerning the maintenance of protein polymorphisms in natural populations.

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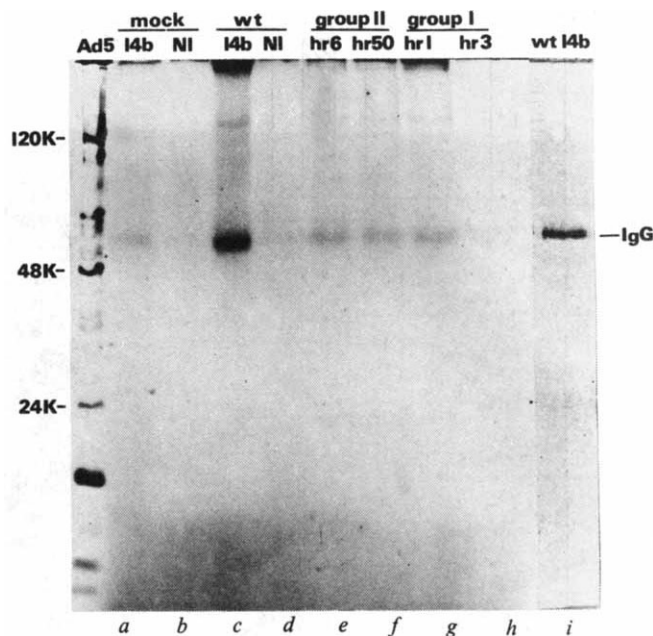
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Immunoprecipitation of protein kinase activity from adenovirus 5-infected cells using antiserum directed against tumour antigens

THE function of transformation proteins coded for by RNA- and DNA-containing tumour viruses is a major question in oncology. A large number of diverse alterations are produced in cells following viral transformation, and such changes are thought to be the result of the action of one or a very few viral gene products. Until recently, very little was known concerning the mechanism of action of such transforming proteins.

Fig. 1 *In vitro* phosphorylation of immunoprecipitates from Ad 5- and mock-infected cells. Proliferating KB cells were infected with Ad 5 (50 plaque-forming units per cell) or were mock infected. At 9 h post-infection, cytoplasmic extracts were prepared and these were immunoprecipitated using a method similar to that of Schaffhausen *et al.*⁹. Cells were scraped off 100-mm Petri dishes, washed twice with cold phosphate-buffered saline, rinsed with cold buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 1 mM CaCl₂, 0.5 mM MgCl₂ and 10% glycerol (v/v), and then lysed in the same rinsing buffer containing 1% NP40 (v/v). After incubating the cells at 4 °C for 20 min, nuclei were removed by centrifugation at 2,000 r.p.m. for 10 min in an IEC centrifuge. To the supernatant from an equivalent of 2×10^7 cells was added 10 μ l of 14b antiserum or nonimmune serum and 40 μ l of protein A-Sepharose CL-4B (Pharmacia) which had been equilibrated in rinsing buffer containing NP40 and glycerol. After 2 h in the cold with constant mixing, the Sepharose beads with bound antibody were pelleted by low speed centrifugation (400 r.p.m. for 30 s in an IEC centrifuge), and then washed 3 times with 100 mM Tris (pH 8.0) containing 200 mM LiCl and 0.1% 2-mercaptoethanol (v/v), and once with 1 mM Tris (pH 7.0) containing 150 mM NaCl. The final pellet was resuspended in 50 μ l of 40 mM sodium acetate (pH 6.0) containing 10 mM MgCl₂ and 7.5 μ M [γ -³²P] ATP (specific activity 250 Ci mmol⁻¹; NEN), and the mixture was incubated for 10 min at 37 °C. The reaction was terminated by the addition of 100 μ l of standard double-strength SDS sample buffer¹² and the immune complexes were eluted from the Sepharose beads by boiling for 2–3 min. The beads were removed by low-speed centrifugation and equal volumes (25 μ l) of supernatant were then analysed by SDS-PAGE using a 16% polyacrylamide slab gel. The gel was stained with Coomassie brilliant blue, dried, and an autoradiograph was prepared using Kodak RP X-Omat film. Mock-infected cells treated with 14b (a) or nonimmune (b) serum. Wild-type Ad 5-infected cells treated with 14b (c) or nonimmune (d) serum. Cells infected with Ad 5 group II host range mutants hr 6 (e) and hr 50 (f) treated with 14b antiserum. Cells infected with Ad 5 group I host range mutants hr 1 (g) and hr 3 (h) treated with 14b antiserum. Coomassie blue-stained gel of wild-type-infected cells treated with 14b antiserum (i). ³⁵S-methionine-labelled Ad 5 virion proteins are included as molecular weight markers.



However, studies carried out by Collett and Erikson with the RNA tumour virus Rous sarcoma virus (RSV) suggested that the RSV transforming protein, src, either possesses or is associated with protein kinase activity¹. Using antiserum directed against virus-coded proteins, it was found that phosphorylation of the heavy chain of the antibody occurred when immunoprecipitates containing src protein were incubated with [γ -³²P]ATP. No such protein kinase activity was observed in immunoprecipitates using uninfected cell extracts, extracts from cells infected with transformation-defective virus, or control nonimmune serum. These data suggest that alterations in protein phosphorylation may regulate RSV transformation. This concept is quite appealing, as protein phosphorylation is already known to have an important regulatory role in several cellular processes^{2–4}. We show here that antiserum directed against tumour antigens of a DNA virus, human adenovirus type 5 (Ad 5), will also immunoprecipitate protein kinase activity. Further, immunoprecipitates from cells infected with transformation-defective host range mutants of Ad 5 contain reduced levels of protein kinase activity.

We have used an approach similar to that of Collett and Erikson to determine if the transforming gene products of Ad 5 possess protein kinase activity. Antiserum against viral transformation proteins was obtained from hamsters bearing tumours induced by Ad 5-transformed hamster cells. The transformed cells, designated 14b, contain 40% of the left end of the Ad 5 DNA molecule⁵ and transcribe 7% of the genome near the left end⁶. This region of the viral DNA molecule is necessary and sufficient for oncogenic transformation^{7,8}. Thus, antiserum prepared against 14b cells should contain antibodies directed against only the transforming proteins of Ad 5.

Cytoplasmic extracts were prepared from Ad 5-infected and mock-infected human KB cells and these were immunoprecipitated with immune or control nonimmune serum, using essentially the procedure of Schaffhausen *et al.*⁹. Washed immune complexes were then incubated in the presence of [γ -³²P]ATP and the samples were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Studies were also carried out using extracts from KB cells infected with host range mutants of Ad 5. These mutants have been shown to fall into two complementation groups, I and II (ref. 10), both of which are transformation defective¹¹.

Figure 1 shows an autoradiograph of immunoprecipitates incubated with [γ -³²P]ATP. With wild-type Ad 5-infected cell extracts and immune serum (Fig. 1c), a major ³²P-labelled species was observed which co-migrated with the heavy chain of IgG (see Fig. 1i). Several minor labelled bands of higher molecular weight were also observed, and additional labelled species appeared with prolonged exposure of the gel. Incorporation into the light chain of the antibody was not detected. Little incorporation was observed with extracts from wild-type-infected cells precipitated by nonimmune serum (Fig. 1d), or with extracts from mock-infected cells (Fig. 1a, b). Counts on bands excised from the gel indicated that the amount of ³²P incorporated into the heavy chain of IgG was 14 times greater with infected cell extracts and immune serum than with controls. Similar experiments with the host range mutants hr 6 and hr 50 of group II (Fig. 1e, f, respectively), and hr 1 and hr 3 of group I (Fig. 1g, h, respectively) gave levels of incorporation into the heavy chain of IgG which were reduced compared to wild-type Ad 5 (30–60% of wild type).

With immunoprecipitates from wild-type-infected cell extracts, the reaction was very rapid, maximum incorporation into IgG occurring within 1 min at 37 °C. No incorporation of ³²P into IgG was observed when the immune complex was heated at 100 °C for 2 min before incubation with [γ -³²P]ATP.

As high levels of endogenous protein kinase activity exist in all mammalian cells, the activity found in immunoprecipitates from Ad 5-infected cells could be due to nonspecific entrapment during formation of the antigen-antibody complex. To exclude this possibility, an unrelated antigen-antibody complex (human IgG and sheep anti-human IgG) was formed in an extract from Ad 5-infected cells. The complex was immunoprecipitated and incubated with [γ -³²P]ATP. No incorporation of ³²P into IgG was observed in these conditions (data not shown). Thus, it is unlikely that the Ad 5-associated protein kinase activity resulted from entrapment of cellular enzymes, as the amount of antigen-antibody complex in this control study was far in excess of that obtained using infected cell extracts and immune serum. Results from another experiment suggest that the protein kinase activity found in immunoprecipitates from infected as opposed to control cell extracts was not due to the presence of vastly different levels of total enzyme activity in the two preparations: cytoplasmic extracts (containing equal amounts of protein) were

incubated with [γ - 32 P]ATP in a reaction mixture similar to that described in the legend to Fig. 1 and the amount of protein kinase activity was measured using endogenous substrates (as described in ref. 12). Ad 5-infected cells contained only 1.4 times more total activity than uninfected cells.

Our results are similar to those obtained with RSV¹. The data suggest that an Ad 5 transformation-specific protein either is a protein kinase or is associated with one. Whether this activity is due to a virus-coded or virus-induced cellular enzyme is at present not clear. However, because the antiserum was raised in hamsters using transformed hamster cells and the immunoprecipitation experiments were carried out with infected human cell extracts, it seems more likely that it is a virus-coded function. The immune antiserum binds specifically to at least two Ad 5-induced polypeptides, of molecular weights of 58,000 and 10,500 (refs 13, 14), as well as other minor species (N.J.L., unpublished). Localisation of protein kinase activity to a specific polypeptide will require further studies. With transformation-defective host range mutants, enzyme activity was reduced, suggesting that these mutants are at least partly deficient in this function. Furthermore, some of the activity obtained with the mutants may be due to leakiness caused by the high multiplicity of infection.

It is now apparent that protein kinase activity is associated with the transforming proteins of both an RNA and a DNA tumour virus and thus alterations in protein phosphorylation may be a general mechanism by which all tumour viruses induce cellular transformation.

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Kinetics of *in vitro* reconstitution of oligomeric enzymes by cross-linking

THE reconstitution of oligomeric enzymes from their denatured state is a consecutive process consisting of folding and association steps. In the case of multimeric enzymes the pathway of reassociation and its correlation with the occurrence of enzymatic activity is an open question. The combined use of cross-linking and SDS-polyacrylamide gel electrophoresis allows the assembly of multimeric enzymes to be followed quantitatively.

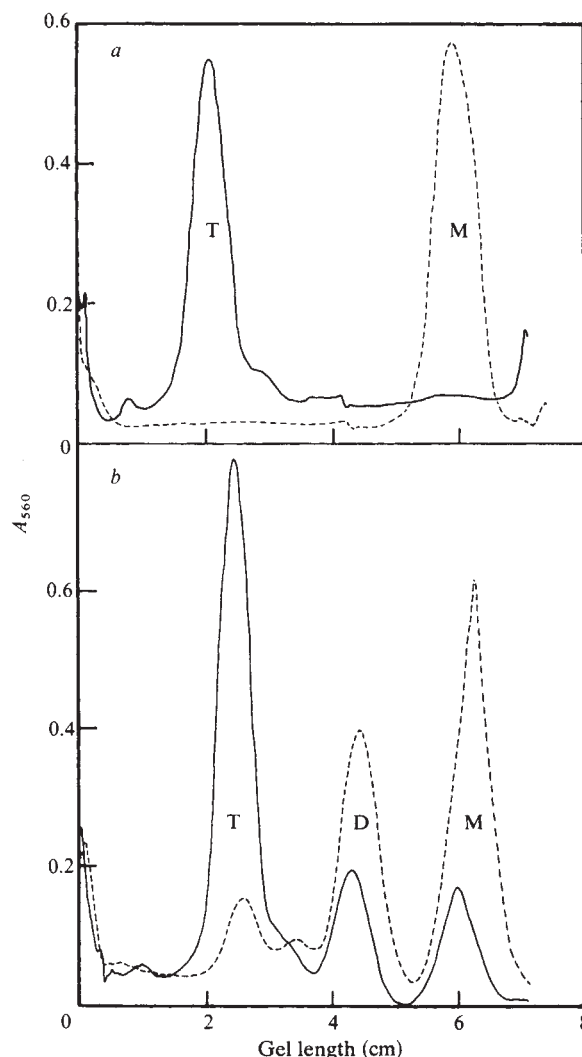


Fig. 1 SDS-polyacrylamide gel electrophoresis of cross-linked LDH-M₄. Cross-linking by 2 min incubation in 0.3–0.4 mM glutaraldehyde, 20 °C, was stopped by addition of 15–25 μ M SDS; unreacted bifunctional reagent was inactivated by 3–4 mM hydrazine. The solutions were heated for 10 min (100 °C) and subsequently stored for 2 h at 20 °C. For concentrating, the protein solutions were dialysed for 24 h against 20% polyethyleneglycol before electrophoresis (5% polyacrylamide gel containing 0.15% SDS). The gels were stained for 2 h with 0.1% Coomassie blue R 250 in 50% trichloroacetic acid, and destained in 7% acetic acid plus 5% methanol. 0.2 M phosphate buffer, pH 7.6, plus 5 mM EDTA was used throughout. The gels were scanned at 560 nm. Correlation of the bands to the tetrameric (T), dimeric (D) and monomeric (M) fractions of LDH was achieved by calibration with proteins of defined molecular weight (dimeric and monomeric human serum albumin, monomeric LDH and lysozyme). *a*, Cross-linking of native tetramers (solid line) and SDS-denatured monomers (dashed line); monomer concentration, 81 nM. *b*, Cross-linking of reactivating LDH after acid dissociation at pH 2.3. Reactivation at 20 °C by dilution at pH 7.6, final monomer concentration, 343 nM. Cross-linking after 210 s (dashed line) and 1 h (solid line) of reactivation and reassociation.

We report here that by applying this technique to a study of lactic dehydrogenase from pig muscle, we have shown that tetramer formation parallels reactivation of the enzyme.

Nascent polypeptide chains fold spontaneously to defined three-dimensional structures, without additional information beyond the specific amino acid sequence of the given protein and its aqueous environment. In addition, protein molecules above a critical chain length tend to associate to form stoichiometrically and geometrically well defined quaternary structures. In this

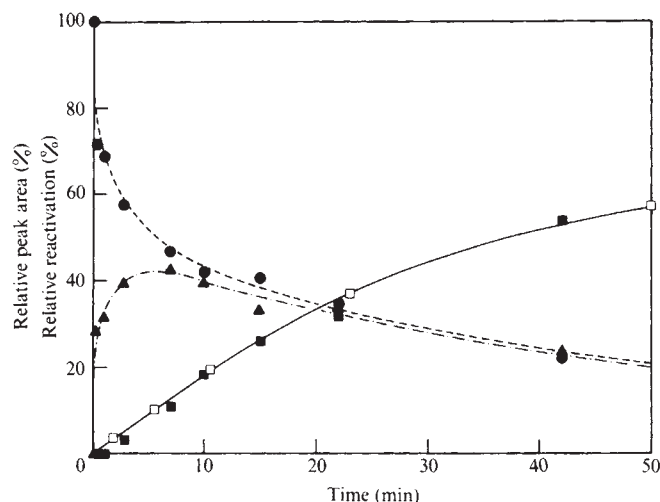


Fig. 2 Determination of the kinetics of reassociation of LDH-M₄ by cross-linking. Experimental details as described in Fig. 1. Reassociation (20 °C) at 170 nM monomer concentration analysed by drawing aliquots at defined times and fixation of the association products by cross-linking. Percentage of monomers (●), dimers (▲) and tetramers (■) defined by the respective peak areas relative to their total amount. Reactivation (□), as determined by testing aliquots at defined times in standard test conditions, was calculated relative to the final extent of reactivation (75% after 6 d).

connection, folding and association can be considered as the final stages of protein biosynthesis. The timing of the two processes must be coordinated because the correct assembly of subunits requires proper folding to defined three-dimensional structures. In this sense the primary function of 'folded monomers' is self-assembly¹.

For single-chain proteins a variety of experimental approaches has allowed the identification of intermediates along the folding pathway². These *in vitro* studies have mostly been aimed at the prediction of three-dimensional structures from the amino acid sequences and the elucidation of the mechanisms dominating the folding process *in vivo*²⁻⁴.

In multi-chain proteins, folding is inherently correlated with the association of subunits. Besides the folding of the isolated polypeptide chains, additional transconformation reactions occur after the association of the native quaternary structure has taken place. Recent analyses of the reactivation kinetics of several oligomeric enzymes have demonstrated that the reassociation process is a prerequisite for conformational transitions leading to the complete restoration of the native enzymatic properties⁵⁻⁸. The corresponding reactivation relaxations obey a simple kinetic mechanism consisting of two consecutive first order and second order reactions^{6,8,9}. The assignment of the respective rate-limiting steps to certain elementary processes in the overall transconformation-association reaction has not been feasible so far. It would require the quantitative determination of the kinetics of association, for example, by light scattering measurements, which is not practicable because of the unfavourable signal-to-noise ratio at low enzyme concentrations and the difficulties connected with a quantitative population analysis.

Therefore, we developed a method of analysing the association kinetics of oligomeric enzymes by cross-linking the refolding subunits with a bifunctional reagent (glutaraldehyde), and subsequent separation of the various association products by SDS-gel electrophoresis. The method allows the detection and quantitative evaluation of intermediates of association even at very low enzyme concentrations. The kinetics of formation and disappearance of these intermediates can be analysed by taking aliquots of the enzyme solution at defined times during the process of reconstitution. Parallel measurements of the

regained enzymatic activity in identical conditions were carried out to correlate the kinetics of reassociation and reactivation. The determination of the kinetic constants of the latter reaction may reveal whether or not subunits as intermediates of reconstitution represent catalytically active entities.

We used lactic dehydrogenase (LDH-M₄) from pig skeletal muscle (Boehringer). The different isoenzymes of mammalian LDH are composed of four subunits, the structural and catalytic properties of which have been investigated in great detail¹⁰. The hydrodynamic and spectral properties of the dissociated, inactive and partially unfolded monomers, present at pH 2.3, have been described previously^{11,12}. After renaturation, the reactivated tetramers were found to be indistinguishable from the native isoenzymes by testing for a variety of physicochemical and enzymatic parameters^{5,11,12}.

The reactivation of both isoenzymes (LDH-M₄ and H₄ from pig) was found to be dominated by second order reactions; slightly sigmoidal relaxations (especially for LDH-H₄) suggest, however, that first order reactions are involved to a certain extent^{8,11-13}. The overall process can be quantitatively described as a consecutive uni-bimolecular reaction sequence. For both isoenzymes, the respective rates of reactivation are not influenced by their coenzyme, NADH or NAD⁺ (refs 5,14).

In the present experiments, LDH-M₄, after denaturation at pH 2.3, was renatured at pH 7.6 in the absence of cofactors or substrates. At specified times after dilution, aliquots were analysed with respect to their particle distribution and the regeneration of enzymatic activity. To ensure fast and quantitative intramolecular cross-linking of associated particles, a high molar excess (10³-10⁷) of glutaraldehyde was used. To prevent intermolecular cross-linking, the reaction was stopped after a constant reaction time of 2 min by addition of 5% SDS and inactivation of excess glutaraldehyde by the addition of hydrazine¹⁵.

As Fig. 1a shows, optimum conditions gave 100% cross-linking and fixation of native enzyme in its tetrameric state, whereas cross-linking of the SDS-dissociated enzyme yielded only monomers. No high molecular weight products were formed by inter-molecular cross bridges in either of the blank experiments. Therefore, cross-linking and subsequent SDS electrophoresis can be used to determine relative amounts of the different states of association present at various times during reassociation (Fig. 1b). The kinetic traces for the decrease of the concentration of monomers and the formation of dimers and tetramers show that within the range of experimental error, tetramer formation clearly parallels the time course of reactivation (Fig. 2).

The identification of monomers, dimers and tetramers during reassociation (Figs 1b, 2) shows that at least two reactions are rate limiting in the overall process of reconstitution. Considering the fact that the reactivation kinetics for LDH-M₄ (apart from an insignificant contribution of rapid first order transconformation reactions) can be described by a single second order rate constant^{8,11}, one may exclude the possibility that two consecutive bimolecular reactions are rate determining for reactivation and tetramer formation. This means that only one of the reactions which is rate limiting for the formation of dimers from monomers, or of tetramers from dimers, is determined by an association process; the other must be of first order¹⁶.

From our data it is not possible to show if the formation of dimers or tetramers is governed by the association reaction. This can only be clarified by analysing the formation of dimers and tetramers at various initial concentrations of monomers. That reactivation and tetramer formation take place together clearly shows that those dimers which accumulate during reassociation do not possess appreciable catalytic activity. This does not rule out the possibility that active dimers occur as intermediates of reactivation. Whether a kinetic model consisting of two consecutive unimolecular and bimolecular reactions is sufficient to describe the observed patterns of dimer and tetramer formation, or whether more sophisticated models, including rapid equilibria, are necessary, cannot yet be decided.

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Formation of a new fluorophore on irradiation of carboxymethylated D-glyceraldehyde-3-phosphate dehydrogenase

BECAUSE of its physiological importance, allosteric properties and ready availability, D-glyceraldehyde-3-phosphate dehydrogenase (G3PDH) is one of the most thoroughly studied of the dehydrogenases. We report here a study of the ligand binding properties of G3PDH. We observed that when active site carboxymethylated G3PDH was irradiated with near ultraviolet light in the presence of NAD^+ , a new fluorophore was formed.

A six-times recrystallised G3PDH from rabbit muscle was modified with iodoacetate until less than 5% of the original activity remained. The reaction mixture was then passed through a Sephadex G-50 column to remove excess iodoacetate. Determination of the sulphhydryl groups present before and after iodoacetate treatment with 5,5'-dithio-bis(2-nitrobenzoic acid) showed that only one sulphhydryl group per subunit was modified—this is presumably Cys-149 at the active site¹. When the active site-carboxymethylated enzyme was irradiated in the presence of excess NAD^+ with a 150-W xenon lamp for 6 min, a new fluorophore was formed. The excitation spectrum of the new fluorophore shows peaks at 290 and 325 nm whereas the emission maximum is at 410 nm (Fig. 1). The photochemical

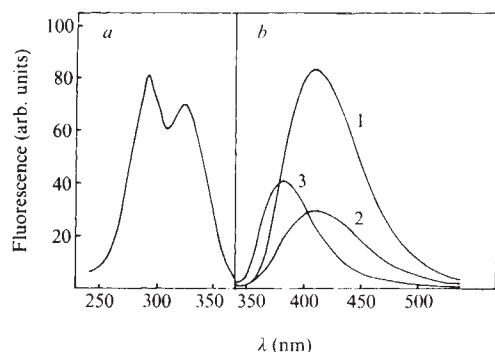


Fig. 1 Excitation and emission spectra of the fluorophores formed on irradiation of carboxymethylated G3PDH with nicotinamide nucleotides. *a*, Excitation spectrum of the fluorophore formed with NAD^+ . *b*, Emission spectra of the fluorophores formed with NAD^+ (1), NADP^+ (2) and NMN^+ (3). Enzyme concentration in all cases, 3 μM .

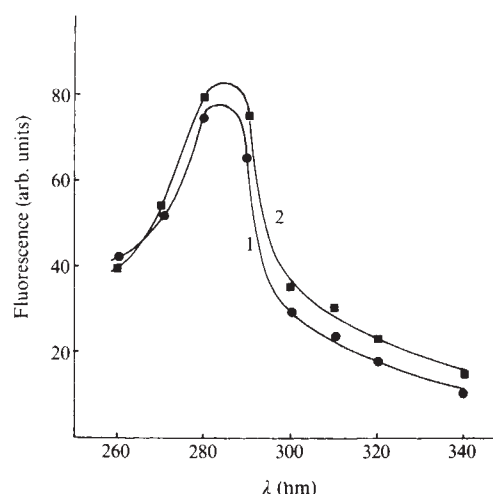


Fig. 2 Photochemical action spectrum for the formation of the NAD^+ fluorophore. Portions of carboxymethylated G3PDH, 3 μM and NAD^+ , 33 μM in 0.1 M phosphate buffer, pH 6.7 were directly irradiated with lights of different wavelengths through the excitation monochromator of a MPF-4 spectrofluorometer at a slit width of 10 nm for 20 min each to give curve 1. The excitation spectrum of a concentrated rhodamine B solution was then scanned. The fluorescence intensity of this solution at each wavelength of excitation is directly proportional to the quantum input at this wavelength and can be used for the correction of curve 1 to give the true photochemical action spectrum for the formation of the new fluorophore (curve 2).

reaction leading to the formation of the new fluorophore appears to be quite specific. Neither the holoenzyme nor the iodoacetamide- or sodium tetrathionate-modified enzyme can form the new fluorophore. At much higher concentrations, NADP^+ and NMN^+ can replace NAD^+ to form new fluorophores with emission maxima at 410 and 385 nm respectively, whereas AMP, ATP or ADPR are completely ineffective. These results suggest that the nicotinamide ring and the carboxyl group introduced at the active site Cys-149 are involved in the formation of the new fluorophore.

The new fluorophore is tightly bound to the enzyme protein as shown by the following experiments. Treatment with excess activated charcoal removed all the NAD^+ added in excess and raised the ratio of A_{280} to A_{260} to 1.4–1.5, but failed to remove the fluorophore from the enzyme protein. After passage through a Sephadex G-50 column, only the protein fraction was fluorescent, the NAD^+ fraction showed no significant fluorescence. Furthermore, ethanol at concentrations greater than 60% also precipitated the enzyme protein together with the fluorophore.

When carboxymethylated G3PDH was irradiated with monochromatic light in the presence of NAD^+ , the photochemical action spectrum obtained (Fig. 2) showed a broad maximum in the 280–290 nm region. Absorption by tyrosine residues decreases sharply at wavelengths longer than 280 nm, so our findings implicate strongly the excitation of tryptophan residues in the formation of the new fluorophore.

With excess NAD^+ removed by charcoal adsorption, the irradiated enzyme shows a broad absorption band at 325 nm due to the new fluorophore. This overlaps with the 295 nm excited protein fluorescence at 335 nm due to tryptophan residues. During the course of irradiation, with the gradual appearance of the new fluorescence band at 410 nm, the tryptophan fluorescence was quenched as shown in Fig. 3. Determinations with *p*-N-dimethylaminobenzaldehyde showed that no destruction of tryptophan residues had occurred. The quenching of tryptophan fluorescence is, therefore, due to a radiationless energy transfer from tryptophan to the new fluorophore. As both the A_{280}/A_{260} ratio and the amino acid composition of the irradiated enzyme seem to suggest that the photochemical formation of the new fluorophore may also be a 'half-of-the-sites' reaction,

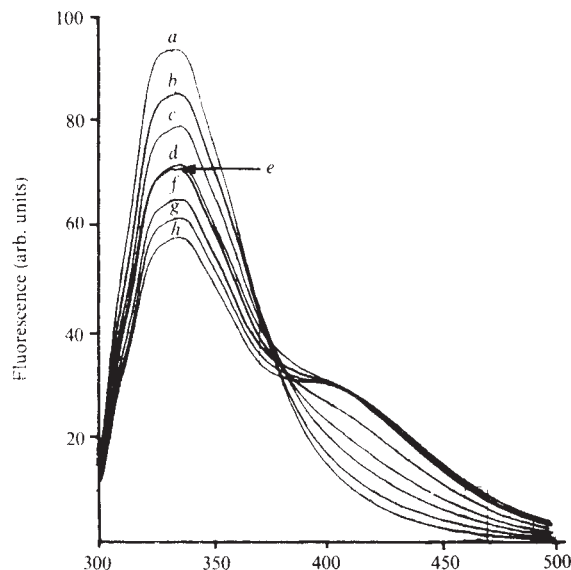


Fig. 3 Quenching of tryptophan fluorescence during the formation of the new fluorophore. Carboxymethylated G3PDH, 3 μ M and NAD^+ 33 μ M in 0.1 M phosphate buffer, pH 6.7 was irradiated in the MPF-4 spectrofluorometer with an exciting light of 290 nm and a slit width of 20 nm. The emission spectra with the exciting wavelength also at 290 nm were scanned at a, 0; b, 1.5; c, 3; d, 6; e, 9; f, 12; g, 15 and h, 20 min.

this energy transfer involves probably only two of the four subunits. Calculations based on the Förster equation with the orientation factor K^2 taken as 2/3 give a distance between tryptophan and the new fluorophore of 15.79 Å. As each subunit of the rabbit muscle enzyme has three tryptophan residues, this probably represents a weighted average value of the distances between the three tryptophan residues to the new fluorophore. This value can be compared with the results of X-ray crystallographic analysis of the lobster³ and bacterial⁴ enzymes.

The formation and the fluorescence of the new fluorophore were found to be sensitive to conformational changes of the enzyme protein. Sodium dodecylsulphate (SDS) at a weight ratio of 1:1 with the enzyme led to a 50% decrease in the fluorescence intensity at 410 nm. However, even more marked

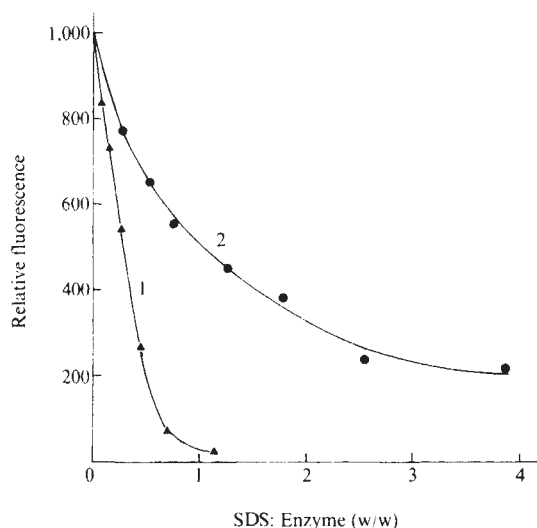


Fig. 4 Effect of sodium dodecylsulphate on the formation and intensity of the new fluorophore. Curve 1 shows the effect of SDS present during irradiation on the formation of the 410 nm fluorophore. Curve 2 shows the effect of SDS added after the formation of the new fluorophore on its fluorescence intensity. Relative fluorescence intensity plotted against the weight ratio of SDS to the enzyme. Final concentration of the enzyme was 2 μ M with 22 μ M NAD^+ in 0.1 M phosphate buffer, pH 6.7.

was the effect of SDS on the formation of the new fluorophore. As shown in Fig. 4, the presence of SDS at a weight ratio of 0.5 during irradiation was enough to decrease the 410 nm fluorescence formed by 75%. It has been reported⁵ that SDS at a weight ratio to G3PDH of 0.5 loosened its tertiary structure and at a ratio of 1 dissociated this enzyme. Our own studies by difference spectroscopy and protein fluorescence also showed that with increasing SDS to enzyme ratios, the internal tryptophan and tyrosine residues of G3PDH were gradually exposed. The great sensitivity of the formation of the new fluorophore seems to suggest that the photochemical reaction leading to its formation has strict requirements on the spatial relationship of excited tryptophan residue(s), the carboxymethyl group introduced at Cys-149 and the nicotinamide ring.

Further studies are needed to establish the chemical nature of the new fluorophore. At present, we can say that it is tightly, probably covalently, bound to the enzyme protein and its spectral and fluorescence properties closely resemble a series of addition compounds⁶ of NAD^+ including NADH.

In spite of some earlier suggestions^{7,8}, very little is known about the role of tryptophan residues in G3PDH. Our results demonstrate the close relationship between tryptophan, Cys-149 and the nicotinamide ring and suggests the need for close examination of this problem.

Further details of this work will be published elsewhere⁹.

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Errata

In the letter 'Photographic amplification of faint astronomical images' by D. F. Malin, *Nature* **276**, 591-593, the figures have been transposed. Figure 1 is shown above Fig. 2 legend and Fig. 2 above Fig. 1 legend.

In the article 'Olivine fractionation equations for basaltic and ultrabasic liquids' by T. H. Pearce, *Nature* **276**, 771-774, equation (12) should read:

$$\log\left(\frac{y}{z}\right) = K \log\left(\frac{x}{z}\right) + \log\left(\frac{y_0}{x_0} \times \frac{z_0^{k-1}}{x_0^{k-1}}\right)$$

equation (14) should read:

$$\log\left(\frac{\text{FeO}}{\text{Al}_2\text{O}_3}\right)_i = K \log\left(\frac{\text{MgO}}{\text{Al}_2\text{O}_3}\right)_i + \log\left(\frac{\text{FeO}}{\text{MgO}}\right)_p \times \left(\frac{\text{Al}_2\text{O}_3}{\text{MgO}}\right)_p^{k-1}$$

In the article 'Analysis of ^{36}Cl in environmental water samples using an electrostatic accelerator' by D. Elmore et al., *Nature* **277**, 22-25, the last sentence in the summary should read: 'For each sample less than 70 mg of AgCl were used, requiring 1-5 litres of water.' Line 19 on page 23 should read 'with mass spectrometers lies in the difficulty of separating...' In line 41 for 'from' read 'with'. In line 47 for ref. 18 read ref. 17. In Table 1 the units in the last three columns should read: (d.p.m. per kg Cl); (mg l^{-1}); ($\times 10^{-6}$, atoms l^{-1}). An extra line should be added to the table:

Groundwater, deep well near Tucson, AZ					
101	990	4	320 ± 50	24	10 53

In line 31 in the right column for 'neutral' read 'natural' and in line 54, for 100-mg read 20-mg.

reviews

Making sense of man's universe

Garrett Hardin

The Little Universe of Man. By C. D. Darlington. Pp. 307. (George Allen and Unwin: London, 1978.) £6.95.

THIS is a biologist's attempt to make sense of the world's past, to foresee its probable future and, by putting foresight into words, possibly to influence what comes.

On what basis shall we appeal to people to alter their actions? "As the teachings of Adam Smith and Robert Malthus, Karl Marx and Charles Darwin agree, the animal principle of the self-interest of individuals, often, as we know, enlightened and extended, is the only motive to be infallibly relied on in the conduct of human affairs". It is psychologically necessary to believe in the rightness of one's action if that action is to be pursued with the fervour required to melt away opposition; out of this necessity springs the paradox of self-interest creating self-deception.

Does self-deception have survival value? It depends on the stage of ecological maturity of society. "As the resources of the earth become more depleted and populations both more crowded and more deprived, the greater will be their effort to pass the problem on to the next generation. Self-deception will become more persuasive. Optimism will take hold. Those who remember 1914 and 1939 will also remember that these smaller calamities were produced by optimists but foreseen by pessimists. Indeed before these events an optimistic temperament had seemed to confer a permanent selective advantage in human evolution. It is not so now".

For the extreme example of an approach that will not solve the world's problems Darlington cites witchcraft: "The profession of witch-doctor dominates Negro life in Africa, creating a religion whose redeeming features we seek in vain". Despite justifiable criticisms that can be levelled against it the European approach to reality is the world's last and best hope: "The world is indepted to Europe for two great achievements which have transformed human life in the last 500 years. One is the control of nature which has led to our present crisis. The other is the understanding of nature and of man which is our means of

escape from this crisis".

At every stage in our progress in understanding ourselves our will to self-deception erects taboos capable of delaying understanding for generations. In the English-speaking world the idea of man as an animal was under a taboo from about 1819 to 1859; human sexuality continued under taboo until about 1930; and the dispassionate discussion of the existence and consequences of innate human differences is still seldom possible. To a geneticist, of course, it is inconceivable that innate, heritable differences could not exist, and it is hard to believe that none of these is humanly important. A Darwinian biologist (and what other can there be?) looks for selection everywhere. Darlington's book is permeated with the Darwinian outlook. It takes only a moment for a perceptive biologist to postulate selective adaptations that may require years to validate. Darlington postulates many, so his book should stimulate many controversies.

The author is not unsympathetic to the currently reigning taboo: "Innate hereditary or genetic differences must not be admitted between individuals or groups, between classes or races, or even between the sexes. But above all what must not be discussed, what must be rejected, are differences in the foundations of human behaviour, the study of brains, of instincts, and of intelligence. These foundations are complex and happily concealed from the public view. They must remain concealed. In a world already overcrowded and over-troubled they might cause more trouble".

A great source of the ever-verdant controversy of nature versus nurture is the awkward fact of causal circularity, sometimes called the "Baldwin

effect." First we select our inventions and then our inventions select us. Equestrian cultures undoubtedly selected for the ability to manage horses, which not all men have. Agriculture and the capital accumulation that it made possible selected for the willingness to engage in sustained, hard work and for a new form of competitiveness between tribes. The increase in the supply of food brought about by agriculture did not set supply and demand permanently in balance; it merely moved the instability to a higher population level and rewarded those who could successfully migrate into new and relatively unexploited environments. Language selected for those who could manipulate language—and their fellow-men—most competently. This fact justifies the overwhelming emphasis on language in intelligence tests; it also explains the passion invested in a particular language. "People have treated the survival of their language in speech and writing as a matter of life and death. And they have been right in doing so since language guarantees the survival of the breeding community".

Trying to make sense of the whole universe of man, as Darlington does, is an ambitious undertaking, scarcely mitigated by calling it a *Little Universe*. Every thoughtful person will find many points of disagreement with the author. The beauty of it is that the points of disagreement are easy to find, for Professor Darlington's exposition is crystal clear. The result is a splendid book for stimulating important discussions. □

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Coral reef research

Coral Reefs: Research Methods. Edited by D. R. Stoddart and R. E. Johannes. Pp. 581. (UNESCO: Paris, 1978.)

THE study of coral reefs, earlier largely sustained by occasional expeditions, sprang suddenly into the fullest vigour in the Marshall Islands in 1945. This was in preparation for the atom bomb

tests that began in the following year in Bikini and then Eniwetok atolls. Preliminary studies were exhaustive in their oceanographical and descriptive ecological content, and as a result of unprecedentedly deep boring, the validity of Darwin's postulates (if not their universal application) was established.

This impetus was sustained by the Coral Atoll Program of the Pacific

Science Board of the National Academy of Sciences of the USA with many of the results appearing in the *Atoll Research Bulletin*. Important guidance was provided in the *Handbook of Atoll Research* published in 1953 by F. R. Fosberg and M.-H. Sachet of the Smithsonian Institution who also edited the *Bulletin*.

Today the subject matter has proliferated widely. The mapping of reefs presents unique problems owing to geographical or seasonal inaccessibility and constantly changing pattern. New methods have developed for drilling, for determination of the sediments into which the reef mass disintegrates and for the local determination of sea level. Evaluation of a fauna and flora living on largely three-dimensional reefs with a territorial fish population presents problems not encountered in temperate seas.

In striking contrast to the surrounding oceanic deserts, atolls are oases of high productivity. But they are fragile systems without any sustaining reservoir of nutrients and phytoplankton, dependent on continuous re-cycling. Everything removed, notably fish, affects total production. The influence of the algal zooxanthellae present in

all hermatypic corals on both calcification and nutrition awaits final evaluation.

Many working on these problems have devised new methods of attack, and new techniques such as aerial photography have appeared. The whole subject has been critically reviewed by Working Group 35 of the Scientific Committee on Oceanic Research of UNESCO which is responsible for this volume with 43 contributions. These are grouped under Morphology and Structure, Biotic Distributions, and Energy and Nutrient Flux, their value the greater because the two first are edited by D. R. Stoddart and the third by R. E. Johannes, respectively the leading workers on the geomorphology and on the productivity of reefs.

Both field workers and those more theoretically interested in the problems of coral reefs will find this book of the greatest help; it will also have permanent value as a record of the current state of enquiry.

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Marine micropalaeontology

Introduction to Marine Micropaleontology. Edited by B. U. Haq and A. Boersma. Pp. 376. (Elsevier: New York and Oxford, 1978.) \$24.

It is fifteen years since the English translation of Pokorný's *Principles of Zoological Micropalaeontology* was published. Since that time there has been no new general text in micropalaeontology, despite the major contribution that the study of microfossils has continued to make in world exploration for oil and gas, and the successive achievements of the Deep Sea Drilling Project. It is perhaps not as well recognised as it ought to be that confirmation of the hypothesis of oceanfloor spreading and plate tectonics in the ocean basins is almost totally dependent on micropalaeontological evidence. Also the general application of 'absolute' ages to marine geological events is heavily dependent on the precision with which micropalaeontological biozones have been related to otherwise relatively isolated radiometric and palaeomagnetic determinations. Latterly too, in association with isotopic analysis, micropalaeontology has begun to yield significant new insights into the periodicity and chemistry of major oceanic changes that are themselves linked to worldwide variations in the planetary environment.

During this period of development knowledge of the various plant and animal microfossil groups has been vastly extended, new numerical methods introduced, and new technologies, such as scanning electron microscopy, adopted. Perhaps it is because of all this endeavour that no micropalaeontologists have had time to compile a review of the current state of their science. Here at last, however, Bilal Haq and Anne Boersma have persuaded a distinguished group of

specialists to contribute to a pre-arranged pattern, a series of up-to-date accounts of the groups which they study. Thus, we have separate chapters on foraminifera, calcareous nannoplankton, ostracodes (by Pokorný himself), pteropods, calpionellids, calcareous algae and bryozoa—all mainly of calcareous composition; radiolaria, diatoms, silicoflagellates—all siliceous; and conodonts, dinoflagellates, spores and pollen and chitinozoa—of phosphatic and organic composition.

Each account gives an outline of the biology, taxonomy and stratigraphical distribution of the respective groups, but more interestingly usually goes on to consider their palaeoecology and contribution to palaeo-oceanic problem-solving. They therefore serve at the same time the prosaic purpose of imparting technical information to the would-be specialist, and also stimulate an interest in problems of more general concern that can be approached by way of micropalaeontology. Naturally with so many different authors contributing the quality is sometimes variable, and, in spite of the extensive review system adopted, the text (be warned) does contain some unexpected errors. Nevertheless the editors are generally to be commended on what they have achieved in terms of uniformity, quality and accuracy. The whole impact of the book is greatly helped by the generous use by all authors of a variety of types of line and half-tone diagrams and illustrations. Here is a text that fully realises that endless prose is not always the best way of imparting information and understanding.

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Chemistry and metabolism of vitamin D

Vitamin D. Edited by D. E. M. Lawson. Pp. 433. (Academic: London, New York and San Francisco, 1978.) £20.50; \$42.40.

UNTIL the late 1960s vitamin D was generally regarded as being a subject of rather academic interest. It was acknowledged to play a role in intestinal calcium transport; and the main symptom of vitamin D-deficiency, a disorder of bone formation, was attributed not to a direct effect of the vitamin but to the secondary disturbance of calcium metabolism.

The past decade has seen dramatic advances in our understanding of the role of this vitamin. It would now be more appropriate to regard it not as

a vitamin but as a metabolite and precursor of a steroid-like hormone. The two hydroxylations, one in the liver and a subsequent one, controlled by parathyroid hormone, in the kidney convert the vitamin to an active form 1,25-dihydroxycholecalciferol. This latter compound acts on intestinal cell nuclei resulting in the induction of synthesis of proteins, including a calcium-binding protein, and leading to an increase in intestinal calcium transport; it is also the most potent stimulator of bone resorption yet discovered. The clinical application of this information has been remarkably rapid. Less than three years after the discovery of the role of the kidney in the intermediary metabolism of vitamin D an appropriate analogue had been synthesised and applied therapeutically by clinicians involved with the management of patients in chronic renal failure.

Despite the frenzy of research activity, particularly over the past five years, we still know remarkably little of the function of the numerous metabolites of vitamin D or their mode of action on target tissues. The complex interrelationship between vitamin D and the other calcium-regulating hormones and the diverse nature of the organs and tissues involved also emphasises the value of a multidisciplinary approach to the research. This book is particularly valuable not only because it provides an excellent review of the current state of our knowledge of vitamin D but also because it serves as such a useful reference source of background information for researchers in various areas. A number of the contributors to this volume are leaders of the research groups responsible for the major advances in vitamin D research and the various chapters therefore provide an authoritative and overall dogmatically balanced account of the literature and of current views on the many controversial aspects of vitamin D.

The chemistry and metabolism of the calciferols is reviewed in two chapters by Bell and by Holick and DeLuca respectively. Three subsequent chapters by Norman, Wasserman *et al.* and by Lawson consider the intestinal system from different aspects. One quarter of

the entire text is devoted to two chapters on bone, by Aaron and by Barnes and Lawson, and a large proportion of these chapters consists of a review of the histology and biochemistry of growth cartilage and bone. Establishing the role of vitamin D in bone formation is clearly one of the main priorities for the next decade and the importance attached to the subject in this book is a reflection of this. The physiology and pathology of vitamin D in man is considered in two more chapters by Stanbury and Mawer, and in the final one Parsons discusses the relationship between it and other calcium-regulating hormones.

Most subjects are covered well but in such a forward-looking book I am surprised that the role of parathyroid and muscle as possible target tissues, and the control of intracellular calcium movement, were not considered in more detail. Overall though this is a valuable contribution to the literature and satisfies a very real need. It will be widely read and appreciated by the large numbers of biochemists, physiologists, nutritionists, clinicians and all others having an interest in calcium and phosphate metabolism.

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Centrifugation techniques

Centrifugation: A Practical Approach. Edited by D. Rickwood. Pp. 224. (Information Retrieval: London and Washington, 1978.) Hardback £8; \$16; paperback £4; \$9.

THE subject matter of this book justifies the publishers' claims that it is an introduction to the technique with an essentially practical basis. It discusses differential pelleting, rate zonal and isopycnic methods and their appropriateness for various purposes with different types of macromolecules, sub-cellular particles and cells. The operation of all types of rotor, including analytical, is described.

There are numerous suggestions for experiments that contribute to the understanding of principles. The comprehensive details provided embrace even the analytical methods to be used after centrifugation. Some of the warnings included imply that the recommended procedures have been modified in the light of experience. Apart from experiments that involve merely the generation or analysis of gradients, expensive equipment is involved. An unsupervised novice would therefore be well advised not to be too ambitious.

On the whole the authors are successful in choosing a happy medium between the risks of overloading the text with qualifying clauses and of presenting oversimplified statements. At some points the grammatical purist may wince, but there are few slips or printers' errors, although "Pedersen" is consistently spelt "Pederson". There is a useful glossary of terms, but symbols are not invariably standard throughout the book.

With two exceptions the number of references at the end of each chapter has a maximum of ten. This restraint is commendable in a book of this nature, but occasionally results in a misleading impression. For instance, the short section dealing with the hydrostatic pressure of gradients contains only recent references and bears no hint that the existence of pressure gradients in any solution being centrifuged has long been recognised.

As there is a paucity of publications covering the same field at an elementary level, this book should find wide acceptance, especially as the price of the paperback should encourage younger workers to have a personal copy.

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Modification of perceptual systems

Perceptual Modification: Adapting to Altered Sensory Environments. By R. B. Welch. (Academic: New York, San Francisco and London, 1978.) \$24.50; £15.90.

THIS excellent book provides the most comprehensive review currently available of research on adaptation of the human senses to rearrangement of the stimulus field. The author has carefully summarised the principal findings of over 500 relevant experiments both published and unpublished, beginning with the late nineteenth century, and including important works completed only during the present year. The research methods used in each experiment are described clearly and succinctly, and are assessed with an objective attitude that is pleasantly refreshing in a field in which evaluative reviews can often be exceedingly biased.

The greater proportion of this book is devoted to examining the diverse types of adaptation to prismatic-induced distortions of the visual field. The various mechanisms that have been proposed to account for the adaptive characteristics of the visual system are presented in detail, and their relative success in explaining the different types of adaptation to visual distortions is reviewed critically. In comparing these mechanisms, the author is careful to make an important distinction between adaptive changes occurring specifically within the visual system and adaptive changes in how vision and movement are coordinated. The failure to make that distinction has often been a source of conflicting results in previous research studies.

The outstanding sections of this book are the reviews on the necessary and sufficient conditions for adaptation to prismatic displacement, adaptation to visual transposition, adaptation to contour distortions, and differences in adaptability among species and individuals. I was particularly impressed by the logical transition from the review of research on adaptation to inversions of the visual field to the review of research on adaptation to small angular rotations of the visual field. His treatment of these topics was notably insightful and informative; a consequence of an exceptionally clear presentation.

The superior chapter in this book, however, is that on curvature adaptation, in which the author effectively contrasts adaptive changes in visual mechanisms used for processing information about objective shape with

those used for processing information about object location. The classical controversy over the role of 'active' as against 'passive' movement in leading to each type of adaptation is also treated most elegantly in that chapter.

If the book has a weakness, it would be the author's commitment to try to make sense out of each and every one of the many diverse findings that he so thoroughly summarises. As he himself points out, there seems to be an almost unlimited number of factors that can contribute to perceptual adaptation. In the final chapter, he proposes a simple theoretical model with the intention being to provide a complete account of perceptual adaptation and thereby resolve numerous controversies in the literature. Although his model does serve well as a descriptive summary of the types of conditions that lead to perceptual adaptation, it really does not provide new insights into what the fundamental underlying processes

might be. My suspicion is that there are many such processes, making an adequate model necessarily more complex than the author acknowledges.

This, however, is a minor criticism; in general, the book is an excellent summary of our current state of knowledge about the extent to which perceptual systems can be modified. It is highly recommended not only as a valuable reference for the professional researcher, but also as an informative introduction for the novice. I think the single most impressive feature of the book is the author's skill at organising an overwhelming number of isolated experiments, so as to provide the reader with a coherent account of one of the most confusing areas of visual perception.

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Catecholamine receptors

Receptor Binding Studies in Adrenergic Pharmacology. By L. T. Williams and R. J. Lefkowitz. Pp. 157. (Raven: New York, \$21.45.

THE proposal by Ahlquist thirty years ago that the responses of tissues to catecholamines are mediated by either α - or β -adrenoceptors has formed the basis of the development of a great number of agonist and antagonist drugs for these receptor sites. It has been somewhat surprising therefore, that despite this rich selection of pharmacological probes, reliable techniques to directly identify catecholamine receptors have only been developed over the past three to four years. However, over this latter period a multitude of research papers have been published describing direct labelling techniques, and substantial advances have been made in our understanding of the characteristics and regulation of these important recognition sites.

The authors of this monograph have played major roles in the development of radiolabelled ligand binding techniques for both α - and β -adrenoceptors. It is therefore to be expected that much of this book describes, in considerable depth, work from their own laboratories. The authors have, however, written useful introductory and linking chapters. There are succinct accounts of the pharmacology of adrenergic receptors, theory of ligand-receptor interactions and a historical perspective reviewing some of the frustrations and disappointments that were inherent in the development of these techniques. The chapter on methodo-

logical approaches contains a few 'tricks of the trade' that may be useful for those embarking on these techniques. However, it was somewhat disappointing that discussion of atypical binding characteristics such as those associated with negative co-operativity and multiple receptor sites, are dealt with only superficially. Nevertheless, the subsequent chapters, devoted to the pharmacological characteristics of α - and β -adrenoceptor binding sites, are particularly detailed and emphasis is rightly placed on the importance of using radiolabelled agonists as well as antagonists.

The development of adrenergic receptor binding techniques has undoubtedly given us a new insight into the way cells may regulate their responsiveness to catecholamines. A chapter devoted entirely to this topic includes a useful account of the theoretical consequences of 'spare receptors' and the intracellular amplification of extracellular signals. Homologous and heterologous hormonal regulation of adrenergic responsiveness are discussed in some depth, though other aspects such as receptor modulation in developing tissue and genetic influences receive less attention.

Although there is no sign that the initial explosive increase in adrenergic receptor research is saturating, this book nevertheless is a timely review and the lucid style of the authors should ensure that it will become a primary and valuable source of information in adrenergic receptor pharmacology.

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